THE IMPACT OF FRESHWATER SALINIZATION ON THE OSMOREGULATORY PHYSIOLOGY OF MAYFLY NYMPHS (*HEXAGENIA RIGIDA*)

FARGOL NOWGHANI

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY
YORK UNIVERSITY
TORONTO, ONTARIO, CANADA

AUGUST 2019

ABSTRACT

Freshwater (FW) salinization (the accumulation of salt in FW), is an environmental issue which threatens the health of FW ecosystems worldwide. This study used FW nymphs of the mayfly *Hexagenia rigida*, to provide a first examination of the effect of salinization on physiological mechanisms of ion transport in this important North American FW invertebrate. Following exposure of mayfly nymphs to sub-lethal levels of salt (7 days in 7.25 g/L NaCl) alterations in morphology and function of ion regulatory organs were found. The enigmatic tracheal gill became an ion secretory organ, instead of acquiring ions as it normally does in FW and the urine producing excretory system of *H. rigida* reduced salt reabsorption rates. Data indicate that ion regulatory "machinery" of *H. rigida* can reorganize in response to low levels of salt contamination, but these alterations are likely to come at a cost because ion transport mechanisms are not functioning normally.

STATEMENT OF CONTRIBUTION

Dr. Jan Coborowski from University of Windsor provided the Ontario Ministry of the Environment, Conservation and Parks (MECP) with *Hexagenia* spp. eggs. The aquatic toxicology unit of MECP provided us with the *Hexagenia* nymphs. Figure 2-3A was a contribution of Lesia Szyca, while the macro image obtained in figure 2-2B was captured by PhD candidate, Chun Chih Chen. Additionally, data displayed in figure 2-6 was analyzed by Chun Chih Chen. Lastly, all chapters were written by Fargol Nowghani and edited by Dr. Andrew Donini, Dr. Scott P. Kelly and Dr. Jean-Paul Paluzzi.

Chapter two has been published and reproduced with permission:

Nowghani, F., Chen, C. C., Jonusaite, S., Watson-Leung, T., Kelly, S. P., & Donini, A. (2019). Impact of salt-contaminated freshwater on osmoregulation and tracheal gill function in nymphs of the mayfly *Hexagenia rigida*. Aquatic Toxicology, 211, 92-104.

ACKNOWLEDGEMENTS

To begin, I would like to express my sincerest gratitude to my brilliant supervisors Dr. Scott P. Kelly and Dr. Andrew Donini. Thank you for always believing in me at times when I didn't even believe in myself. I will forever be grateful of the guidance, support and encouragement I was given, which made me into the researcher I am today and the leader I strive to be in the future. I will forever be grateful for the roles in which you have played in my academic as well as personal journey.

I would like to express my appreciation to my advisor, Dr. Jean-Paul Paluzzi for his time, insightful guidance and comments as well as for generously allowing me to use his laboratory resources to complete my research.

I would also like to thank my mentor and good friend Dr. Sima Jonusaite, who took me under her wing as a new researcher. Her trust in me, endless patience for my mistakes and exceptional research skills largely influenced my love for research. I am grateful for having someone to look up to such as yourself and strive to be as good in my job one day as you are in yours.

Additionally, my experience at York would not have been complete had it not been for the amazing and inspiring members of the Kelly lab and Donini lab. I thank you all, past and present members for your friendliness, encouragement and guidance throughout the years. First and foremost, thank you Sophie for always putting a smile on my face and in my heart, you are by far my favourite animal. Dennis, thank you for encouraging me to do a masters and for your continual assistance throughout the years. Lidiya, thank you for always providing help and sharing your knowledge about the endless techniques you knew in the laboratory. Gil, thanks for all the encouragement and support you have provided and your sense of humour! Thank you, Chun, for being a very knowledgeable scientist, I would not have completed my masters without your endless help and support. Elia, thank you for your encouragement and support throughout the years. Ana, thank you for your brilliant ideas, optimism and endless support, the greatest ideas only arose when I brainstormed with you. Thank you, Andrea, not only for dragging me to the gym but for always extending a helping hand and for providing insightful comments and guidance.

Thank you to the members of the Paluzzi lab for all the support, motivation and the good memories.

Lastly, I owe a special thanks to my amazing family, which I feel so lucky to be a part of, for always supporting me and motivating me to do my best.

TABLE OF CONTENTS

LIST OF TABLES

Chapter Two:

LIST OF FIGURES

Chapter Two:

Chapter Three:

CHAPTER 1:

OVERVIEW

1.1 Summary

As a result of human related activities, freshwater (FW) salinization has become a growing global issue. Although this problem is acknowledged there still remains a lack of action by policy makers to regulate the inflow of salt contaminants into our freshwater systems. In this chapter I discuss (a) the largest contributor to FW salinization, being the winter de-icing agent (road salt) typically applied in the form of NaCl and the mechanism through which this contaminant causes damage to our ecosystems. In addition, I discuss (b) the potential risk that NaCl imposes on the physiology of FW insects, discussing the major ionoregulatory organs and the impact of salt contamination on their function. Lastly, I (c) review the role Ephemeroptera nymphs play as good research models for highlighting the negative impact of FW salinization on FW insects in general. Overall, this review is sought to provide readers with fundamental knowledge needed to better understand the significance of my thesis and why I formulated the hypotheses and objectives that underpin the experiments that I conducted.

1.2 The threat of salt-contaminated water

The global issue of FW contamination with salts reduces aquatic biodiversity and threatens human health (Vörösmarty et al., 2010). Toxic pollutants such as pesticides and heavy metals, which are a result of anthropogenic activities such as mining, agriculture and industrial waste are often regulated to limit their environmental effects (Schuler et al., 2018). However, less toxic pollutants such as phosphates and salts are not regulated as well and due to increased anthropogenic activities (i.e. urbanization), their rate of accumulation in FW has been increasing exponentially over the past couple of decades (Lofgren, 2001; Corsi et al., 2010; Cañedo-Argüelles et al., 2016). Salts used as de-icing agents during winter months are applied to roadways to melt ice and reduce vehicle accidents (Fay and Shi, 2012). Once applied, high concentrations of these salts enter surrounding bodies of water such as lakes, rivers, streams or underground water through runoff. Road salts include sodium

chloride (NaCl), magnesium chloride ($MgCl_2$), and calcium chloride ($CaCl_2$). Globally however, sodium chloride is the most common and cost-efficient roadway de-icing agent (Dugan et al., 2017). With a rapid surge in road-salt application alongside other human-related activities, FW salinization imposes a serious threat to ecosystem functions, fisheries and drinking water quality, all of which negatively affect human health (Dugan et al., 2017; Schuler et al., 2018). In more extreme cases, salinized drinking water can directly affect human wellbeing by causing hypertension (Khan et al., 2011). While the visible damaging effects of road salt can be seen on rusting cars (Acharya et al., 2018) and dying plants (Devitt et al., 2014), the impact of FW salinization on aquatic animals is not as well acknowledged. There remains a lack of progress in protecting FW ecosystems from salinization, which is probably driven by the absence of risk acknowledgment by the public and policy makers. By supporting future research in this field, insects can be used as tools to fill in the gap in knowledge about the mechanism in which toxicity affects the physiological parameters of FW aquatic life.

1.3 Osmoregulatory strategies in FW species

FW insect larvae are hyperosmoregulators and maintain a constant osmotic and ionic concentration in the haemolymph that is higher than the surrounding water. Having a hyperosmotic haemolymph and being permeable to salts and water puts these animals at risk for excess water gain and passive ion loss (Maloiy, 1979). To overcome this, insects tightly regulate the removal of excess water and the intake and retention of ions through the integrated function of osmo/ionoregulatory organs (Beyenbach and Piermarini, 2009). For example, the alimentary canal actively transports solutes and nutrients from ingested material into the haemolymph (Shaw and Stobbart, 1963). Here, Malpighian tubules (MTs), which are blind-ended diverticula of the alimentary canal arising between the posterior midgut and the anterior hindgut, are major ionoregulatory organs that aid in urine formation (Patrick and Bradley, 2000). The number and morphology of MTs vary across different

species of insects (Landa, 1969; Gaino and Rebora, 2000; Jarail and Scudder, 1970). In some insects, functional regionalization can be seen, as the most distal segment of the tubule is involved in secretion and the proximal segments are involved in reabsorption (O'Donnell and Maddrell, 1995). In other insects, secretion occurs along the whole length of the tubule (Bradley et al., 1982). Furthermore, variation in the fine structure of the tubules is too great across insect species to be generalized. However, all insect MTs share the function of secreting fluid which is considered to be the primary urine. Further modifications are made to the primary urine (making it hypoosmotic in the case of FW aquatic insects) at the rectum and this fine tunes ion and fluid handling to meet the homeostatic needs of the animal (Ramsay, 1950). Having low osmotic permeability, the rectum conserves ions with minimal water flow, resulting in a dilute urine. In this manner, ions are conserved, making sure the haemolymph stays osmotically stable and excessive hydration is prevented (Wigglesworth, 2012).

Although this intricate system tightly regulates ion and water levels in the body fluids, other studies have reported additional ion-uptake sites in select FW insects (Edwards and Harrison, 1983; Flishie and Campbell, 1984; Komnick, 1977; Komnick and Wichard, 1975; Nguyen and Donini, 2010). For instance, the anal papillae, which are postanal cutaneous appendices (in midge and mosquito larvae) or preanal evaginations of the rectal wall (in blackfly larvae) have been found in many Dipteran as well as caddisfly larvae (Komnick, 1977). Having mostly been studied in mosquito and chironomid larvae, the anal papillae is known to participate in active ion (Na^+, Cl^- and K^+) uptake in dilute environments (Credland, 1976; Donini and O'Donnell, 2005; Koch, 1938; Nguyen and Donini, 2010). Moreover, changes in external salinity result in changes in papillae size and ultrastructure (Sohal and Copeland, 1966; Kefford et al., 2011). For example, mosquito larvae reared in FW typically have larger anal papillae with more apical plasma membrane infolds and mitochondria, relative to larvae reared in high salinity water (Sohal and Copeland, 1966). In the same light, ionocytes (i.e. specialized salt transporting cells that were formerly referred to as 'chloride

cells') have also been discovered in the epidermis of three insect orders; Ephemeroptera, Plecoptera, and Heteroptera (Wichard et al., 1973; Kapoor and Zachariah, 1973; Komnick and Wichard, 1975). In Ephemeroptera, ionocytes have been localized as dense populations on tracheal gills, which are external extensions of the tracheal system that are utilized for gas exchange in this group (Wichard and Komnick, 1971). The apical openings of ionocytes are regions of modified cuticle which allows the movement of ions through the otherwise fairly impermeable cuticle (Filshie and Campbell, 1984). Interestingly, the abundance, ultrastructure and function of these cells have been reported to be altered by changes in environmental salinity (Wichard et al., 1973), leading (in part) to the idea that gill ionocytes possess an ionoregulatory function. More recently, this has been confirmed by directly measuring ion flux rates across the tracheal gill of a mayfly nymph (Nowghani et al., 2019). Thus, put together, we can conclude that the FW insect larvae maintain homeostasis through the coordinated function of the excretory system comprised of the MTs and the hindgut, as well as additional sites of ion-uptake such as the anal papillae or tracheal gills.

1.4 Ephemeroptera nymphs as tractable research models

Ephemeroptera, commonly known as mayflies are the oldest extant winged insects, dating back to the Carboniferous and Permian periods (Brittain, 1982). A fairly small order, mayflies contain just over 2000 species and are found in most FW environments around the world (Hubbard and Peters, 1976), including North American Great Lakes. An interesting feature of mayflies is their life cycle, which is comprised of an aquatic nymph stage, lasting between 1-2 years and a two-form (imago and subimago) terrestrial adult stage lasting between 2-3 days (Clifford, 1982). We know that mayflies are able to maintain haemolymph homeostasis in dilute saline environments, but this also means that these organisms display a low tolerance for increased water salt content (Silberbush et al., 2005). This makes them particularly susceptible to the problem of salinization. More specifically, a species of mayflies known as *Hexagenia rigida* are particularly susceptible to haloclines (*i.e.* salt gradients) in

contaminated standing water because they are sedimentary/burrowing nymphs (Brittain, 1982). Mayfly species such as *Hexagenia* have long been recognized as bioindicators of FW quality and have been used in water quality control tests and soil toxicity testing (Burks, 1953; Howland et al., 2019; Bartlett et al., 2018). Given the lack of knowledge in mayfly physiology, it can be difficult to interpret the resulting survival patterns of these species to toxicants. Thus, more emphasis must be placed on the physiological, molecular and cellular biology of mayflies in order to gain insight into their osmoregulatory physiology and the effects of toxicants, in particular, salts. Lastly, the influence of salt contamination on the ability of these organisms to maintain salt and water balance will contribute significantly to a broader understanding of the deleterious effects of FW salinization in North America.

1.5 Hypothesis and objectives

The current study sought to address the current gap in knowledge of mayfly ion and water regulatory physiology, using *H. rigida* as a model for examining the deleterious effects of FW salinization. It can be hypothesized that important ionoregulatory organs such as the MTs, hindgut and tracheal gills will significantly alter their function to acclimate to changes in environmental salinity under sub-lethal conditions.

To consider this idea further, I had three objectives as follows:

1. To examine effects of salt contamination of FW (using NaCl) on physiological endpoints of ion and water regulation by looking at haemolymph ion levels and body water content.
2. To examine the effects of salt (NaCl) contamination of FW on tracheal gill function by looking at the biochemical, ion transport, morphological and ultrastructural properties of the tracheal gill epithelium.
3. To investigate the ionoregulatory contribution of the MTs and rectum in the whole-body response of *H. rigida* to salt (NaCl) contaminated water.

These studies provide much needed insight into salt and water balance of FW mayfly nymphs and act as a link between the field of physiology and ecology. This has the potential to inform regulators of the importance of controlling salinization of FW ecosystems.

1.6 References

Acharya, S., Sheladiya, M., & Acharya, G. D., 2018. Preventive measures for corrosion of deicers to metals: need of the hour. CORCON 2018.

Bartlett, A.J., Hedges, A.M., Intini, K.D., Brown, L.R., Maisonneuve, F.J., Robinson, S.A., Gillis, P.L., de Solla, S.R., 2018. Lethal and sublethal toxicity of neonicotinoid and butanolide insecticides to the mayfly, *Hexagenia* spp. Environmental pollution, 238, 63-75.

Beyenbach, K. W., & Piermarini, P. M. (2009). Osmotic and ionic regulation in insects. Osmotic and ionic regulation: cells and animals, 231-293.

Bradley, T. J., Stuart, A. M., & Satir, P. (1982). The ultrastructure of the larval Malpighian tubules of a saline-water mosquito. Tissue and Cell, 14(4), 759-773.

Brittain, J. E. (1982). Biology of mayflies. Annual Review of Entomology, 27(1), 119-147.

Burks, B. D. (1953). The mayflies, or Ephemeroptera, of Illinois. Illinois Natural History Survey Bulletin; v. 026, no. 01.

Cañedo-Argüelles, M., Hawkins, C. P., Kefford, B. J., Schäfer, R. B., Dyack, B. J., Brucet, S., … & Coring, E. (2016). Saving freshwater from salts. Science, 351(6276), 914-916.

Clifford, H. F. (1982). Life cycles of mayflies (Ephemeroptera), with special reference to voltinism. Quaestiones Entomologicae, 18(1), 15-90.

Corsi, S. R., Graczyk, D. J., Geis, S. W., Booth, N. L., & Richards, K. D. (2010). A fresh look at road salt: aquatic toxicity and water-quality impacts on local, regional, and national scales. Environmental Science & Technology, 44(19), 7376-7382.

Credland, P. F. (1976). A structural study of the anal papillae of the midge *Chironomus riparius* Meigen (Diptera: Chironomidae). Cell and tissue research, 166(4), 531-540.

Devitt, D. A., Wright, L., Landau, F., & Apodaca, L. (2014). Deicing salts; assessing distribution, ion accumulation in plants and the response of plants to different loading rates and salt mixtures. Environment and Natural Resources Research, 4(1), 73.

Donini, A., & O'Donnell, M. J. (2005). Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. Journal of Experimental Biology, 208(4), 603-610.

Dugan, H. A., Bartlett, S. L., Burke, S. M., Doubek, J. P., Krivak-Tetley, F. E., Skaff, N. K., … & Roberts, D. C. (2017). Salting our freshwater lakes. Proceedings of the National Academy of Sciences, 114(17), 4453-4458.

Edwards, H. A., & Harrison, J. B. (1983). An osmoregulatory syncytium and associated cells in a freshwater mosquito. Tissue and Cell, 15(2), 271-280.

Fay, L., & Shi, X. (2012). Environmental impacts of chemicals for snow and ice control: state of the knowledge. Water, Air, & Soil Pollution, 223(5), 2751-2770.

Filshie, B. K., & Campbell, I. C. (1984). Design of an insect cuticle associated with osmoregulation: the porous plates of chloride cells in a mayfly nymph. Tissue and Cell, 16(5), 789-803.

Gaino, E., & Rebora, M. (2000). The duct connecting Malpighian tubules and gut: an ultrastructural and comparative analysis in various Ephemeroptera nymphs (Pterygota). Zoomorphology, 120(2), 99-106.

Howland, J. R., Alexander, A. C., Milani, D., Culp, J. M., & Peru, K. M. (2019). Effects of oil sands process water mixtures on the mayfly *Hexagenia* and field-collected aquatic macroinvertebrate communities. Ecotoxicology, 1-11.

Hubbard, M. D., & Peters, W. L. (1976). The number of genera and species of mayflies (Ephemeroptera)[Insects]. Entomological News.

Jarial, M. S., & Scudder, G. G. E. (1970). The morphology and ultrastructure of the Malpighian tubules and hindgut in *Cenocorixa bifida* (Hung.) (Hemiptera, Corixidae). Zeitschrift für Morphologie der Tiere, 68(4), 269-299.

Kapoor, N. N., & Zachariah, K. (1973). A study of specialized cells of the tracheal gills of *Paragnetina media* (Plecoptera). Canadian Journal of Zoology, 51(9), 983-986.

Kefford, B. J., Reddy-Lopata, K., Clay, C., Hagen, T., Parkanyi, O., & Nugegoda, D. (2011). Size of anal papillae in chironomids: Does it indicate their salinity stress? Limnologica, 41(2), 96-106.

Khan, A. E., Ireson, A., Kovats, S., Mojumder, S. K., Khusru, A., Rahman, A., & Vineis, P. (2011). Drinking water salinity and maternal health in coastal Bangladesh: implications of climate change. Environmental health perspectives, 119(9), 1328-1332.

Koch, H. J. (1938). The absorption of chloride ions by the anal papillae of Diptera larvae. Journal of experimental Biology, 15(1), 152-160.

Komnick, H. (1977). Chloride cells and chloride epithelia of aquatic insects. In International Review of Cytology, v. 49, 285-329. Academic Press.

Komnick, H., & Wichard, W. (1975). Chloride cells of larval *Notonecta glauca* and *Naucoris cimicoides* (Hemiptera, Hydrocorisae). Cell and Tissue Research, 156(4), 539-549.

Landa, V. (1973). A contribution to the evolution of the order Ephemeroptera based on comparative anatomy. In Proceedings of the first international conference on Ephemeroptera Brill, Leiden, 155-159.

Löfgren, S. (2001). The chemical effects of deicing salt on soil and stream water of five catchments in southeast Sweden. Water, Air, and Soil Pollution, 130(1-4), 863-868.

Maloiy, G. M. O. (1979). Comparative physiology of osmoregulation in animals. Academic Press.

Nguyen, H., & Donini, A. (2010). Larvae of the midge *Chironomus riparius* possess two distinct mechanisms for ionoregulation in response to ion-poor conditions. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 299(3), R762-R773.

Nowghani, F., Chen, C. C., Jonusaite, S., Watson-Leung, T., Kelly, S. P., & Donini, A. (2019). Impact of salt-contaminated freshwater on osmoregulation and tracheal gill function in nymphs of the mayfly *Hexagenia rigida*. Aquatic Toxicology, 211, 92-104.

O'Donnell, M. J., & Maddrell, S. H. (1995). Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female *Drosophila*. Journal of Experimental Biology, 198(8), 1647-1653.

Patrick, M. L., & Bradley, T. J. (2000). The physiology of salinity tolerance in larvae of two species of Culex mosquitoes: the role of compatible solutes. Journal of Experimental Biology, 203(4), 821-830.

Ramsay, J. (1950). Osmotic regulation in mosquito larvae. Journal of Experimental Biology, 27(2), 145-157.

Schuler, M. S., Cañedo-Argüelles, M., Hintz, W. D., Dyack, B., Birk, S., & Relyea, R. A. (2018). Regulations are needed to protect freshwater ecosystems from salinization. Philosophical Transactions of the Royal Society B, 374(1764), 20180019.

Shaw, J., & Stobbart, R. H. (1963). Osmotic and ionic regulation in insects. In Advances in insect physiology, Vol. 1, 315-399. Academic Press.

Silberbush, A., Blaustein, L., & Margalith, Y. (2005). Influence of salinity concentration on aquatic insect community structure: a mesocosm experiment in the Dead Sea Basin Region. Hydrobiologia, 548(1), 1-10.

Sohal, R. S., & Copeland, E. (1966). Ultrastructural variations in the anal papillae of *Aedes aegypti* (L.) at different environmental salinities. Journal of insect physiology, 12(4), 429-434.

Vörösmarty, C. J., McIntyre, P. B., Gessner, M. O., Dudgeon, D., Prusevich, A., Green, P., … & Davies, P. M. (2010). Global threats to human water security and river biodiversity. Nature, 467(7315), 555.

Wichard, W., & Komnick, H. (1971). Electron microscopial and histochemical evidence of chloride cells in tracheal gills of mayfly larvae. Cytobiologie, 3(2), 215-228.

Wichard, W., Tsui, P. T. P., & Komnick, H. (1973). Effect of different salinities on the coniform chloride cells of mayfly larvae. Journal of Insect Physiology, 19(9), 1825-1835.

Wigglesworth, V. B. (2012). The principles of insect physiology. Springer Science & Business Media.

CHAPTER 2:

IMPACT OF SALT-CONTAMINATED FRESHWATER ON OSMOREGULATION AND TRACHEAL GILL FUNCTION IN NYMPHS OF THE MAYFLY *HEXAGENIA RIGIDA*

2.1 Summary

The impact of freshwater (FW) salinization on osmoregulation as well as tracheal gill morphology and function was examined in nymphs of the mayfly *Hexagenia rigida* following exposure to salt contaminated water (SCW, 7.25 g/L NaCl) for a 7-day period. Ionoregulatory homeostasis was perturbed in SCW exposed *H. rigida* nymphs as indicated by increased haemolymph Na^+, K^+ and Cl^- levels as well as haemolymph pH and water content. Despite this, SCW did not alter gill Na^+-K^+-ATPase (NKA) or V-type H^+-ATPase (VA) activity. In addition, NKA and VA immunolocalization in gill ionocytes did not show alterations in enzyme location or changes in ionocyte abundance. The latter observation was confirmed using scanning electron microscopy (SEM) to examine exposed tracheal gill ionocyte numbers. Ionocyte surface morphometrics also revealed that SCW did not change individual ionocyte surface area or ionocyte fractional surface area. Nevertheless, analysis of Na^+ movement across the tracheal gill of mayfly nymphs using scanning ion-selective electrode technique indicated that FW nymphs acquired Na^+ from surrounding water, while tracheal gills of SCW nymphs had the capacity to secrete Na^+. Because Na^+ secretion across the gill of SCW-exposed animals occurred in the absence of any change in (1) NKA and VA activity or (2) ionocyte numbers/surface exposure, it was reasoned that Na^+ movement across the gill of SCW animals may be occurring, at least in part, through the paracellular pathway. The ultrastructure of tracheal gill septate junctions (SJs) supported this idea as they exhibited morphological alterations indicative of a leakier pathway. Data provide a first look at alterations in osmoregulatory mechanisms that allow *H. rigida* nymphs to tolerate sub-lethal salinization of their surroundings.

2.2 Introduction

Salinization of freshwater is occurring globally through human activities such as mining, industrial processing, agriculture and winter de-icing of roads, sidewalks and parking lots (Cañedo-

Argüelles et al., 2016; Kaushal et al., 2017; Barros et al., 2012). In the case of de-icing agents, sodium chloride (NaCl) is the most common and cost-efficient roadway de-icer in temperate regions of North America and Europe (Dugan et al., 2017; Fay and Shi, 2012). Road salt is applied as rock salt or brine and in addition to de-icing, it can also be used to pre-treat roads and prevent ice formation (Fay and Shi, 2012). However, because de-icing road salt quickly dissolves, it can be transported into surrounding freshwater (FW) through leaching and/or runoff (Dugan et al., 2017), and in this regard de-icing road salt is acknowledged to contribute significantly to the salinization of FW lakes (Chapra et al., 2008; Novotny et al., 2008; Likens and Buso, 2010; Mueller and Gachter, 2011; Swinton et al., 2015), rivers and streams (Thunqvist, 2004; Kaushal et al., 2005; Kelly et al., 2008; Corsi et al., 2010), as well as groundwater (Williams et al., 2000; Panno et al., 2006; Howard and Maier, 2007). Indeed, recent studies have predicted that de-icing road salt contamination of Northern temperate lakes (which represent the highest density of lakes on the planet) will result in many of these FW bodies exceeding the aquatic life criterion for chronic chloride exposure (as outlined by the US Environmental Protection Agency) in a matter of decades (Dugan et al., 2017). This is a grave situation because healthy FW ecosystems support diverse living organisms, the majority of which would never experience an increase in environmental salinity during the course of their life cycle. Therefore, in addition to considering the survival threshold of FW organisms in the face of salt contamination, it is imperative to understand how the physiology of FW organisms alters in response to sub-lethal salt contamination levels, in particular because physiological alterations are likely to have broad consequences (Dugan et al. 2017).

The life cycle of a mayfly is dominated by the nymph stage, which is an early life aquatic phase that typically resides in clean FW, although some can inhabit brackish water (Chadwick and Feminella, 2001). Nevertheless, the mayfly nymph, like other FW insect larvae, are considered to be stenohaline, in that they can only tolerate a relatively narrow range of salinity. These animals are

osmoregulators and in this regard they possess a haemolymph that is hypertonic to FW and a suite of osmoregulatory strategies that allow them to combat haemodilution from osmotic influx of water and diffusive loss of ions (Sutcliffe, 1962; Jonusaite et al., 2011; Nowghani et al., 2017). These strategies are not conducive to inhabiting salt water and stenohaline FW aquatic insects display different sensitivities to salt. Using commercially available salts which are designed to closely resemble the ionic composition of seawater, a study showed that mayflies (Ephemeroptera) were the most sensitive to salt compared with other stenohaline insect groups which included beetles (Coleoptera), true bugs (Hemiptera), damselflies and dragonflies (Odonata), stoneflies (Plecoptera), caddisflies (Trichoptera) and flies (Diptera) (Kefford et al., 2012). Furthermore, the species richness and abundance of mayflies decrease with salination of freshwater (Kefford et al., 2011; Pond, 2010; Pond et al., 2008; Beermann et al., 2018). For example, an assessment of mined watersheds showed a loss of Ephemeroptera taxa and this result was strongly correlated with water conductivity; however, the anionic sources were primarily HCO_3^-/CO_3^{2-} and SO_4^-, rather than Cl^- which is the primary source of anions in areas with winter road maintenance (Pond et al., 2008; Cormier et al., 2013). A recent study examining the effect of multiple stressors on macroinvertebrate communities showed that increasing NaCl concentration alone resulted in a decline of Ephemeroptera abundance (Beermann et al., 2018). Therefore, evidence shows that among stenohaline insects, mayflies are particularly sensitive to salination of freshwater regardless of the ionic composition and perhaps the fundamental reason for this observation is that mayflies cannot maintain salt and water balance at salt concentrations in which other stenohaline insects still can.

Mayfly nymphs possess an external extension of the tracheal system that forms tracheal gills that may serve respiratory as well as osmoregulatory function (Wichard and Komnick, 1971; Wichard et al., 1973; Nowghani et al., 2017). In general, tracheal gills run along both sides of the lower abdomen but show a great deal of morphological diversity which may be a consequence of the habitat in which

the particular mayfly species nymphs reside. For example, members of Prosopistomatidae possess gills that are concealed and reside in a mixture of sand and gravel in areas of flowing water (Ghee, 2004). Members of Baetidae typically reside on rocks and boulders in faster flowing water and possess plate like lamellate gills (Ghee 2004; Wichard et al., 1973). Sediment burrowing mayflies like *Hexagenia rigida* (Ephemeridae) have feather-like gills (Saouter et al., 1991; Nowghani et al., 2017). Furthermore, the importance of mayfly nymph gills for acquiring oxygen was shown to relate to the oxygen content of the habitat for the species examined (Wingfield, 1939). It was shown that gills of *Baetis* species which inhabit relatively well oxygenated flowing waters do not aid oxygen consumption whereas those of sediment burrowing mayflies aid oxygen consumption even if the nymphs are placed in well oxygenated water (Wingfield, 1939). Therefore, caution is required in generalizing the findings of gill function from a particular mayfly species to all mayflies. Nevertheless, it would appear that despite these differences in habitat and gill morphology the gills possess putative salt transporting cells that exhibit ultrastructural characteristics typical of ionocytes and these cells have previously been referred to as "ephemerid chloride cells" (Wichard and Komnick, 1971; Wichard et al., 1973). Moreover, recent studies using *Hexagenia rigida* nymphs have shown that tracheal gill ionocytes possess an enriched complement of two major ion transporting enzymes, Na^+-K^+-ATPase (NKA) and V-type H^+-ATPase (VA) and that *H. rigida* nymph gills acquire Na^+ directly from surrounding FW (Nowghani et al. 2017). Indeed, the degree of Na^+ acquisition is substantial compared to other externally exposed ionoregulatory organs of insect larvae (e.g. anal papilla), and taken together, evidence suggests that tracheal gills contribute significantly to the regulation of salt and water balance in FW *H. rigida* nymphs (Nowghani et al., 2017). However, nothing is known about tracheal gill function in *H. rigida* following an alteration in environmental conditions, and in particular how they might respond to a change in water ion content. It has been reported that nymphs of *Callibaetis coloradensis* acclimated to either dilute or concentrated FW

exhibit differences in the number of tracheal gill ionocytes as did *C. floridans* collected from either brackish water or FW (Wichard et al., 1973). In addition, some aspects of the fine structure of ionocytes were also reported to differ between animals acclimated to varying water ion conditions (Wichard et al., 1973). These observations support the idea that tracheal gill function can be impacted by water ion content, but how salinity or salinization impacts overall salt and water balance of mayfly nymphs or ion transport across tracheal gills of these animals has yet to be examined.

This study aimed to address a knowledge gap in our understanding of mayfly physiology by using *H. rigida* as a model to examine the effects of FW salinization on systemic salt and water balance as well as tracheal gill structure and function. Based on the observations of Wichard et al. (1973) and more recent observations of tracheal gill ion transport capacity (Nowghani et al., 2017), it can be hypothesized that *H. rigida* nymphs will be capable of adjusting their physiology in order to acclimate to sub-lethal concentrations of salt in part, by (1) remodeling the morphology and (2) adjusting the function of tracheal gills. These hypotheses were tested by exposing *H. rigida* nymphs to FW contaminated with salt (NaCl) and then; (1) examining the gill for alterations in ultrastructure and biochemical endpoints of ion transport as well as (2) testing for functional alterations in gill ion transport by directly measuring Na^+ transport at the gill surface.

2.3 Materials and Methods

2.3.1 Animals

Nymphs of the mayfly *H. rigida* (16–35 mm in length) were obtained from a culture maintained by the Ontario Ministry of the Environment, Conservation and Parks (OMECP, Etobicoke, ON, Canada). The culture is supplemented on yearly basis with the collection of *H. rigida* and *H. limbata* eggs by Dr. Jan Ciborowski's laboratory in Windsor, Ontario, Canada. OMECP culture conditions for *Hexagenia* spp. are defined by standard operating procedures (Ontario Ministry of the Environment and Climate Change, 2016). Following acquisition, nymphs were maintained in

aquaria containing dechlorinated municipal tap water (approximate composition in μmol l^{-1}: [Na^+] 590; [Cl^-] 920; [Ca^{2+}] 760; [K^+] 43; pH 7.35) and uncontaminated field-collected control sediment (8 cm depth; also supplied by the OMECP; chemistry available upon request) in a 3:1 ratio. Water was aerated, water temperature was maintained at 21°C and aquaria were exposed to a 12 h light:12 h dark photoperiod cycle. Twice a week a 10 ml aliquot of solution containing cereal grass Media (Ward's Science, Rochester, NY, USA; 37.5 mg ml^{-1} dechlorinated water) and ground TetraFin Goldfish Flake Food (Tetra Holding US, Blacksburg, VA, USA; 25 mg ml^{-1} dechlorinated water) was added to each aquarium.

2.3.2 Exposure of nymphs to salt contaminated water (SCW):

NaCl, 7.25 g l^{-1} (BioShop Canada Inc.) was dissolved in dechlorinated municipal tap water and this is referred to as salt contaminated water (SCW) throughout this study (see composition above for dechlorinated water). Salt levels in SCW were confirmed by measuring Cl^- according to Zall et al., (1956). Size matched larvae (~20 mm in length) were directly transferred to 2 L containers containing Lake Erie sediment (~ 7.6 cm deep) and either FW or SCW. Animals were exposed to either FW control conditions or SCW for a period of 7 days under conditions as outlined above. At the end of the 7 day exposure samples of FW and SCW media were collected from the treatment tanks and Cl^- was measured according to Zall et al. (1956) while Na^+, K^+ and H^+ activities were measured with ion selective microelectrodes as described in section 2.3 below with results presented in Table 1.

Table 2-1: Ion levels ($mmol^{-1}$) and pH values for treatment medium (FW and SCW).

Ions	Freshwater	Salt-contaminated water
Na^+	5.3 ± 0.07	120.7 ± 3.7
K^+	0.8 ± 0.03	0.3 ± 0.03
Cl^-	4.7 ± 0.4	133.4 ± 3.8
pH	8.6 ± 0.02	8.5 ± 0.03

2.3.3 Haemolymph collection and Na^+, K^+, Cl^- and pH analysis:

Following removal from experimental water, nymphs were given a quick rinse with distilled water (to remove any residual salts) and carefully blotted with a Kimwipe™ to remove residual moisture. The animals were then immobilized on a Sylgard® (Dow Corning, Mississauga, ON, Canada) filled glass petri dish base following which the cuticle was punctured at an articular membrane at the base of a leg. This allowed a pool of haemolymph to form which was collected using a micropipette. One aliquot of collected haemolymph was then transferred to an oil dish for immediate examination of Na^+, K^+, and H^+ activity while a second aliquot was transferred to a solution of 1% nitric acid in de-ionized water until further analysis of Cl^-. Haemolymph [Cl^-] was determined using a colourimetric technique (Zall et al., 1956). The activity of Na^+, K^+ and H^+ was determined using ion-selective microelectrodes (ISME). For ISME, the following ionophore cocktails (Fluka, Buchs, Switzerland) and back-fill solutions (in parentheses) were used: K^+ ionophore I cocktail B (100 mmol l^{-1} KCl), Na^+ ionophore II cocktail A (100 mmol l^{-1} NaCl) and H^+ ionophore I cocktail B (100 mmol l^{-1} NaCl/100 mmol l^{-1} sodium citrate, pH 6.0). The ISMEs were calibrated in the following solutions: K^+: 1.5 mmol l^{-1} KCl/298.5 mmol l^{-1} NaCl and 15 mmol l^{-1} KCl/285 mmol l^{-1}NaCl; Na^+: 30 mmol l^{-1} NaCl/270 mmol l^{-1} LiCl and 300 mmol l^{-1} NaCl; H^+: 150 mmol l^{-1} NaCl containing 1 mmol l^{-1} Hepes, pH 7.5 and 8.5. The ISME slopes (mV) for a 10-fold change in ion concentration in FW samples were (means±s.e.m.): 48.9±2.44 (N=6) for K^+ and 60.7±1.0 (N=5) for Na^+. The ISME slopes (mV) for a 10-fold change in ion concentration in SCW samples were (means±s.e.m.): 55.3±1.9 (N=4) for K^+ and 57.6±1.34 (N=4) for Na^+. To measure pH, standard buffer solutions of pH 7 and 10 were used where the slope of the ISME was 140.1±12.9 (N=3) for FW samples and 106.4±1.1 (N=4) for SCW samples. An ML165 pH Amp connected to a PowerLab 4/30 (ADInstruments, Colorado Springs, CO, USA) data acquisition system was used to measure voltage readings and LabChart 6 Pro software (ADInstruments) was used for analysis. Haemolymph ion activities were calculated

using the following equation, as described by Donini et al., (2007):

Equation 2-1: $a_h = a_c \times 10^{\Delta V/S}$

Where a_h is the haemolymph ion activity, a_c is the ion activity in one of the calibration solutions, ΔV is the difference in voltage between the haemolymph and the calibration solution, and S is the slope of the electrode measured in response to a 10-fold change in ion activity. Equation 1 was modified for pH by substituting 1000 for 10, as well as utilizing the slope over a 1000-fold change in ion activity.

2.3.4 Measurement of body water content:

Whole body water content was determined gravimetrically. Nymphs were rinsed to remove any sediment from the outer body surface and then blotted dry on tissue paper. The wet weight of a nymph was recorded after placing it into a pre-weighed microcentrifuge tube. The tube (with cap open) was then placed in an oven at 60°C for 24 h. Following this, the tube containing the dried animal was weighed. All weights were obtained using a high precision UMX2 Automated-S microbalance (Mettler Toledo). Total body moisture content (expressed as %) was then calculated as follows:

Equation 2-2: *Body moisture content (%)* = $[(Wt_w - Wt_d)/Wt_w] \times 100$

Where Wt_w is the weight of the animal before drying and Wt_d is the weight of the animal after drying.

2.3.5 Na^+-K^+-ATPase (NKA) and v-type H^+-ATPase (VA) activity measurements:

Six pairs of tracheal gills arise perpendicular to the abdomen along either side of *H. rigida* nymphs. For enzyme analysis, all gill filaments were removed from the nymph abdomen at their proximal ends and placed in microcentrifuge tubes before being flash frozen in liquid nitrogen. Because of the small size of this organ, tracheal gills from 3 nymphs were pooled in a tube for an *n* of 1 before freezing. During dissection, tubes were held on ice. Gill samples were stored at −80°C

until use. NKA and VA activities were measured according to methods outlined in detail by (McCormick, 1993) and modified by Jonusaite et al., (2011). Enzyme inhibitor concentrations for NKA and VA were 5 mmol l^{-1} of ouabain and 1 μmol l^{-1} of bafilomycin respectively, in accordance with those reported by Nowghani et al., (2017).

2.3.6 Immunohistochemical localization of NKA and VA:

The lower abdomen containing the tracheal gills of FW and SCW nymphs was isolated and fixed in Bouin's solution (overnight, room temperature). Fixed tissues were further processed, embedded, and sectioned (4 μm thick) as outlined by Chasiotis and Kelly, (2008). Immunohistochemical localization of NKA was carried out using a mouse monoclonal antibody raised against the α-subunit of avian NKA (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) at 1:10 dilution, as described by Jonusaite et al., (2013). To visualize NKA, Alexa Flour 594-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used at a 1:400 dilution. The localization of VA was achieved using a guinea pig polyclonal antibody raised against the V_1 complex of the VA of *Manduca sexta* (1:5000; kind donation from Dr. Weiczorek, University of Osnabruk, Germany). To visualize VA, Alexa Fluor 488-conjugated goat anti-guinea pig antibody (1:400; Jackson ImmunoResearch Laboratories) was used. Tissues were rinsed in PBS and mounted in ProLong Gold Antifade reagent (Invitrogen Canada, Burlington, ON, Canada). An Olympus IX81 inverted microscope (Olympus Canada, Richmond Hill, ON, Canada) equipped with an X-CITE 120XL fluorescent Illuminator (X-CITE, Mississauga, ON, Canada) was used to capture images. Images were processed using ImageJ software (ImageJ ver.1.51J8, National Institutes of Health, USA).

2.3.7 Measurement of Na^+ flux across the tracheal gills:

Scanning Ion-selective Electrode Technique (SIET) was used to determine Na^+ voltage gradients at the tracheal gill surface so that Na^+ flux rates could be calculated. The SIET measures

ion flux with micrometer spatial resolution in real time and is therefore useful in determining site specific ion fluxes from individual organs and large cells (Donini and O'Donnell, 2005; Kolosov et al., 2018). Na^+ voltage gradients were determined spatially along the central axis of one individual tracheal gill selected from the first pair (most anterior) of gills on the abdomen of each animal. Voltage gradient measurements were recorded in 6 zones of equal length (region I – VI) from the proximal (I) to distal (VI) region of the gill as outlined in a previous study (see Nowghani et al., 2017 and Fig. 2-3A in the current study). To do this, the gill was first anchored into a drop of petroleum jelly (Hargell Inc., Toronto, ON, Canada) and then placed in a solution-filled petri dish. For FW nymphs the petri dish was filled with a solution of dechlorinated water (composition as reported above). For SCW-exposed nymphs, the petri dish was filled with a solution made up of 0.5 mmol l^{-1} NaCl and 206.5 mmol l^{-1} N-methyl-D-glucamine (NMDG). This allowed the osmolarity of the solution to closely resemble SCW. A description of the SIET system and protocol utilized in this study has been provided in detail by Donini and O'Donnell, (2005). When calibrated in 0.1, 1 and 10 mmol l^{-1} solutions of NaCl and NMDG (for total of 207 mmol l^{-1} ionic strength), the Na^+ ISME slope (mV) for a 10-fold change in ion concentration for the FW bath was (60.8±1.8, N = 4) and (61.6±1.4, N = 6) for the SCW bath.

2.3.8 Calculation of Na^+ flux rates from SIET-measured voltage gradients:

The following equation was used to obtain concentration gradients from voltage gradient readings collected using ASET 2.0 software:

$$\textbf{Equation 2-3}: \Delta C = C_B \times 10^{\Delta V/S} - C_B,$$

Where ΔC is the concentration gradient between the two points measured in μmol l^{-1} cm^{-3}; C_B is the background ion concentration, calculated as the average of the concentration at each point measured in μmol l^{-1}. ΔV is the voltage gradient obtained from ASET in μV; and S is the slope of the

electrode. After obtaining the concentration gradients, a flux value for the corresponding gradients was calculated based on Fick's law of diffusion with **Equation 2-3**:

Equation 2-4: $J_I = D_I(\Delta C) / \Delta x$,

Where J_I is the net flux of the ion in pmol cm^2 s^{-1}; D_1 is the diffusion coefficient of the ion ($1.92\times10^{-5}cm^2$ s^{-1} for K^+, 1.55 x 10^{-5} cm^2 s^{-1} for Na^+); ΔC is the concentration gradient in pmol cm^{-3}; and Δx is the distance between the two points measured in cm.

2.3.9 Electron microscopy and image analysis:

Tracheal gill samples prepared for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were the first two (anterior most) pairs of gill filaments on the abdomen (i.e. 4 gill filaments). For SEM morphometric analysis, 5 animals were sampled from each treatment.

For SEM, detached whole gill filament samples were fixed overnight at 4°C in a solution of 2% glutaraldehyde (Electron Microscopy Sciences, PA, USA) in 0.1M phosphate buffer (pH 7.2). Samples were then dehydrated using graded acetone solutions (30 – 100%) and final drying was achieved using anhydrous tetramethylsilane (2 x 10 min; Sigma-Aldrich, ON, Canada). Samples were mounted and a Leica EM ACE200 instrument (Leica Microsystems) was used for sputter deposition (Au/Pd, 15 nm thickness). SEM images were then acquired using a XL30 environmental scanning electron microscope (FEI, Hillsboro, OR, USA). Tracheal gill ionocyte surface morphometrics were determined as described by Chasiotis et al. (2011). All image analysis was carried out using ImageJ image analysis software (ImageJ ver.1.51J8, National Institutes of Health, USA).

For TEM, gill samples were fixed overnight in 2% glutaraldehyde (Electron Microscopy Sciences, PA, USA) and 4% paraformaldehyde (BioShop, ON, Canada) prepared in an insect saline solution (composition in mmol l^{-1}: 5 KCl, 74 NaCl, 1 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 8.6 HEPES, 20 glucose, 10 glutamine, pH 7.0; adapted from Leonard et al. (2009)). Fixed samples were then post-fixed in 1% osmium tetroxide and dehydrated in a graded ethanol series followed by propylene oxide.

Samples were embedded in Quetol-Spurr resin, following which 90 nm sections were cut using a Leica EM UC7 ultramicrotome. Sections were stained with uranyl acetate and lead citrate and viewed using a FEI Tecnai 20 TEM.

2.3.10 Statistics:

All data are expressed as mean values ± s.e.m. (n), where n is the number of biological replicates. A Student's *t*-test was used to compare FW (control) and SCW groups. For tracheal gill ionocyte morphometrics, a two-way ANOVA was used, where the independent variables were water salt content (FW versus SCW) and gill region (proximal versus distal). To compare Na^+ flux measurements that were acquired spatially along the gill (i.e. regions I – VI, see Fig. 3A), a one-way ANOVA was used. Following examination of data using ANOVA, and where applicable, a Tukey's comparison test was used. Data were considered to be significantly different when $P<0.05$. All statistical analyses were conducted using SigmaStat 3.5 software (Systat Software, San Jose, CA, USA).

2.4 Results

2.4.1 Haemolymph ion composition and body water content:

Mortality of nymphs in FW and SCW treatments for 7 days did not exceed 10%. Exposure to SCW resulted in a significant increase in haemolymph Na^+, Cl^- and K^+ (Fig. 2-1A). Mean values for haemolymph Na^+ were 96.8 ± 4.9 mmol l^{-1} (n = 18) and 157.2 ± 2.5 mmol l^{-1} (n = 21) for FW and SCW nymphs, respectively, while Cl^- levels were 75.7 ± 2.2 mmol l^{-1} (n = 18) for FW and 110.7± 2.8 mmol l^{-1} (n = 18) for SCW (Fig. 2-1A). Haemolymph K^+ were 6.5 ± 0.54 mmol l^{-1} (n = 18) in FW animals and 7.84 ± 0.3 mmol l^{-1} (n = 21) in SCW animals (Fig. 2-1A). Haemolymph pH was also found to have increased in nymphs residing in SCW. Values were 7.9 ± 0.1for FW animals and 8.1 ± 0.02 for SCW animals (Fig. 2-1A). Finally, an increase in body water content was observed in SCW-acclimated nymphs in comparison to FW nymphs (Fig. 2-1B).

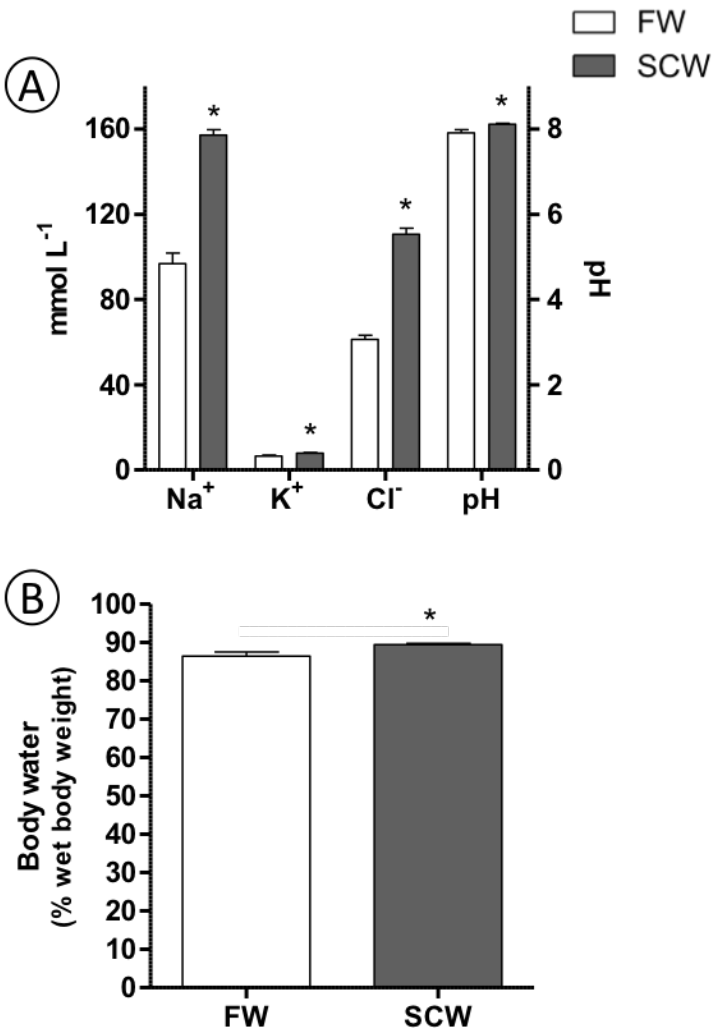

Figure 2-1: The effect of salt-contaminated water (SCW) on (A) haemolymph ion concentrations and pH as well as (B) whole body water content of mayfly (*Hexagenia rigida*) nymphs. Control animals were held in freshwater (FW). All data are expressed as mean values ± s.e.m., n = 23. An asterisk denotes a statistically significant difference ($P<0.05$) between FW and (SCW)-animals as determined by a Student's *t*-test.

2.4.2 Gill NKA and VA activity and immunolocalization:

NKA and VA activities in the tracheal gills of nymphs acclimated to FW or SCW were not significantly different. NKA activity of FW animals was 1.6 ± 0.1 μmol ADP/mg protein/hour while NKA activity of tracheal gills taken from SCW-acclimated animals was 1.2 ± 0.2 μmol ADP/mg protein/hour (Fig. 2-2A). For VA, activities were 0.7± 0.2 and 0.9 ± 0.4 μmol ADP/mg protein/hour for FW and SCW-acclimated animals, respectively (Fig. 2-2A).

NKA and VA were found to be abundant in select cells of the tracheal gills irrespective of water composition (Fig. 2-2B,C). Most commonly, NKA and VA were observed in the same cell, and under such circumstances, NKA immunoreactivity (ir) appeared to be basolateral while VA-ir was apical (Fig 2D-G). In some cases, only NKA- or VA-ir were observed in a cell (Fig. 2-2D-G). Following acclimation to SCW, there was no clear alteration in the pattern of NKA and VA fluorescence when compared to samples taken from FW animals (Fig. 2-2D-G). That is, the distribution, number and staining patterns of NKA and VA in tracheal gill cells appeared similar in the two treatments.

2.4.3 Na^+ transport across tracheal gills of FW and SCW nymphs:

Na^+ transport was examined spatially along the gills of mayfly nymphs, at six different regions in a proximal to distal orientation (Fig. 2-3A). The gills of FW nymphs absorbed Na^+ from surrounding water across all 6 regions measured. A modest but significantly greater rate of Na^+ absorption was seen across the first 4 gill regions (from the proximal end) versus the last two regions at the distal end of the structure (Fig. 2-3B). In contrast the gills of SCW nymphs were found to secrete Na^+ across all 6 regions examined, and spatial differences in secretion rates were pronounced (Fig. 2-3C). More specifically, the first two regions from the proximal end exhibited far greater secretion rates than the last 4 regions of the gill (Fig. 2-3C).

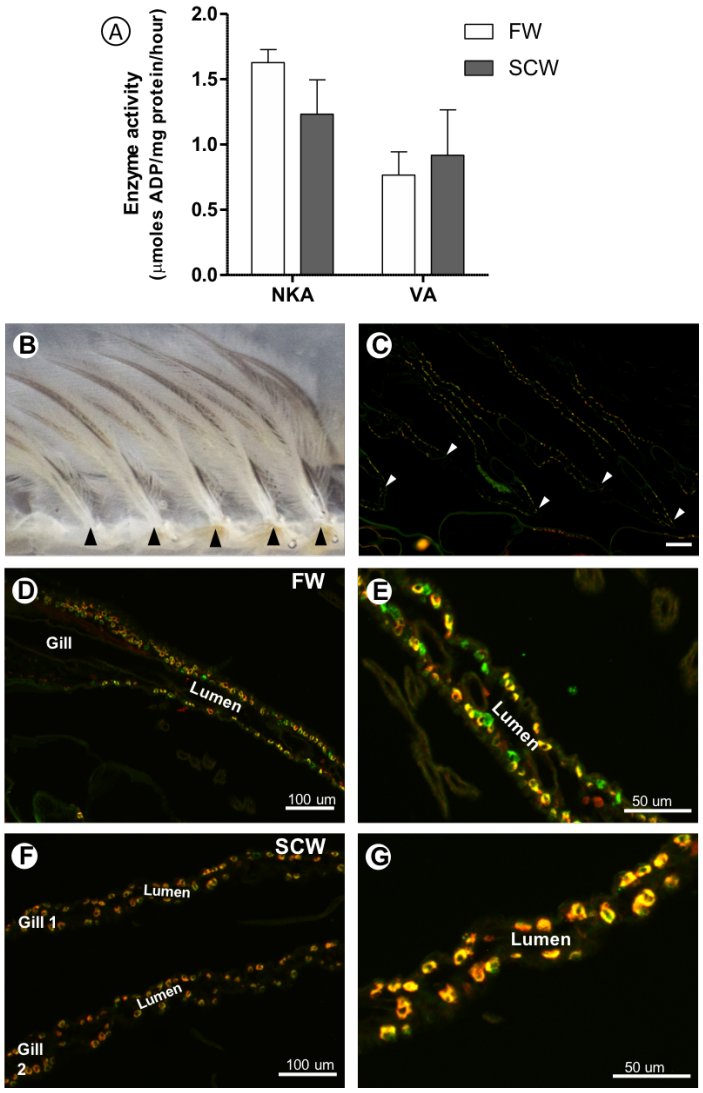

Figure 2-2: The effect of salt-contaminated water (SCW) on (A) gill Na^+-K^+-ATPase (NKA) and V-type H^+-ATPase (VA) activity as well immunolocalization of NKA (red) and VA (green) in the tracheal gill of (B-E) freshwater (FW) control and (F,G) SCW -acclimated mayfly (*Hexagenia rigida*) nymphs. In panel (A) data are expressed as mean values ± s.e.m.; n=3-5. SCW acclimation had no significant effect on NKA or VA activity in the tracheal gill. Panel (B) shows an image of the abdomen at the proximal region of tracheal gill attachment (indicated by black arrowheads). This provides orientation for panel (C) which shows a lower magnification image of sectioned tracheal gills immunostained for NKA and VA. A white arrowhead in panel (C) indicates the proximal end of a tracheal gill where it is attached to the abdomen. Panel (D) is a close up image of NKA and VA immunostaining in the tracheal gills of a FW nymph, with panel (E) being a magnified image of (D). Panel (F) is a close-up image of NKA and VA immunostaining in the tracheal gills of a SCW-acclimated nymph, with panel (G) being a magnified image of (F). Scale bars in panels (B) and (C) = 200 µm. Scale bars in panels (D-G) = 50 µm.

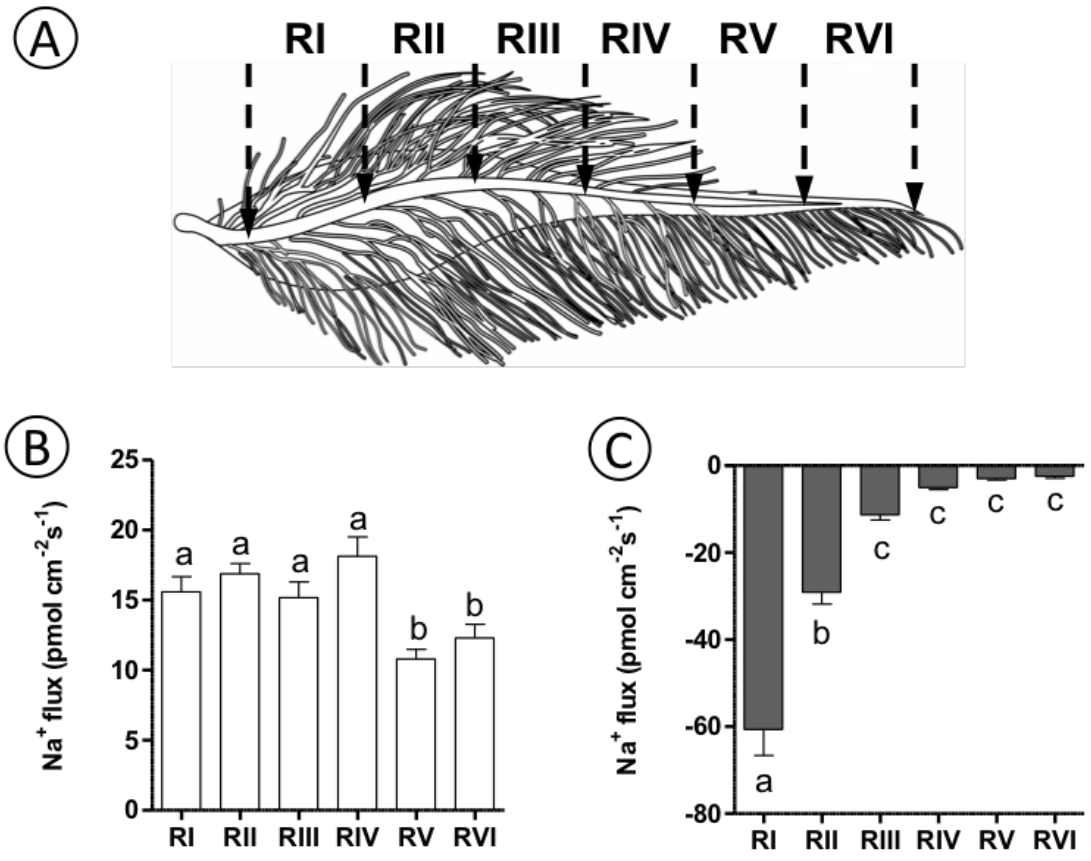

Figure 2-3: Scanning ion-selective electrode technique measurement of Na^+ flux rates along (A) 6 regions of the tracheal gill filament central axis, RI – RVI of (B) freshwater (FW) control and (C) salt-contaminated water (SCW) -acclimated mayfly (*Hexagenia rigida*) nymphs. RI is the proximal region of the tracheal gill near the point of attachment to the abdomen. In panels (B) and (C), all data are expressed as mean values ± s.e.m., n = 18. Letters denote a significant difference ($P<0.05$) between regions as determined by a one-way ANOVA followed by a multiple comparison test.

2.4.4 Ultrastructural analysis of tracheal gill ionocytes:

By examining the surface ultrastructure of the tracheal gill, SEM and TEM analysis revealed the presence of cuticular modifications associated with apical regions of putative ionocytes. SEM observation showed that these regions of apical ionocyte exposure were observed along the central axis (proximal and distal regions) of the tracheal gills of both FW (Fig. 2-4A-C) and SCW- (Fig. 2-4D,E) acclimated nymphs. No evidence of ionocyte exposure could be seen at the surface of the secondary filaments (i.e. at the surface of the hair-like extensions along the margins of the gill). The surface ultrastructure of a gill ionocyte manifested as a defined, circular, and smooth cuticular region and each one of these appeared to be quite uniform irrespective of their spatial position on the gill (Fig. 2-4B,C). This same ultrastructure was observed on the gill surface of animals acclimated to SCW (Fig. 2-4D,E). To confirm that the apical structures being observed were cuticular regions overlying ionocytes, TEM was used to capture sectioned images of the tracheal gill (Fig . 2-4F). These images showed that the only region of the gill where the cuticle was not a morphologically uniform band ~ 650 – 800 nm thick (see Fig. 2-5A), were regions overlying cells that exhibited clear ionocyte hallmarks (see Fig. 2-4F, Fig. 2-5B). More specifically, these cells (ionocytes) were voluminous and exposed to the surrounding environment via a modified region of cuticle (Fig. 2-4F, Fig. 2-5B, C). In addition, ionocytes were found to contain numerous mitochondria (Fig. 2-5D) and a tubular system that appeared to be a complex invagination of cell membrane (Fig. 2-5E). Furthermore, the cuticular region overlying the gill ionocyte exhibited an ultrastructure that was indicative of it being porous (i.e. a porous plate) (Fig. 2-5C).

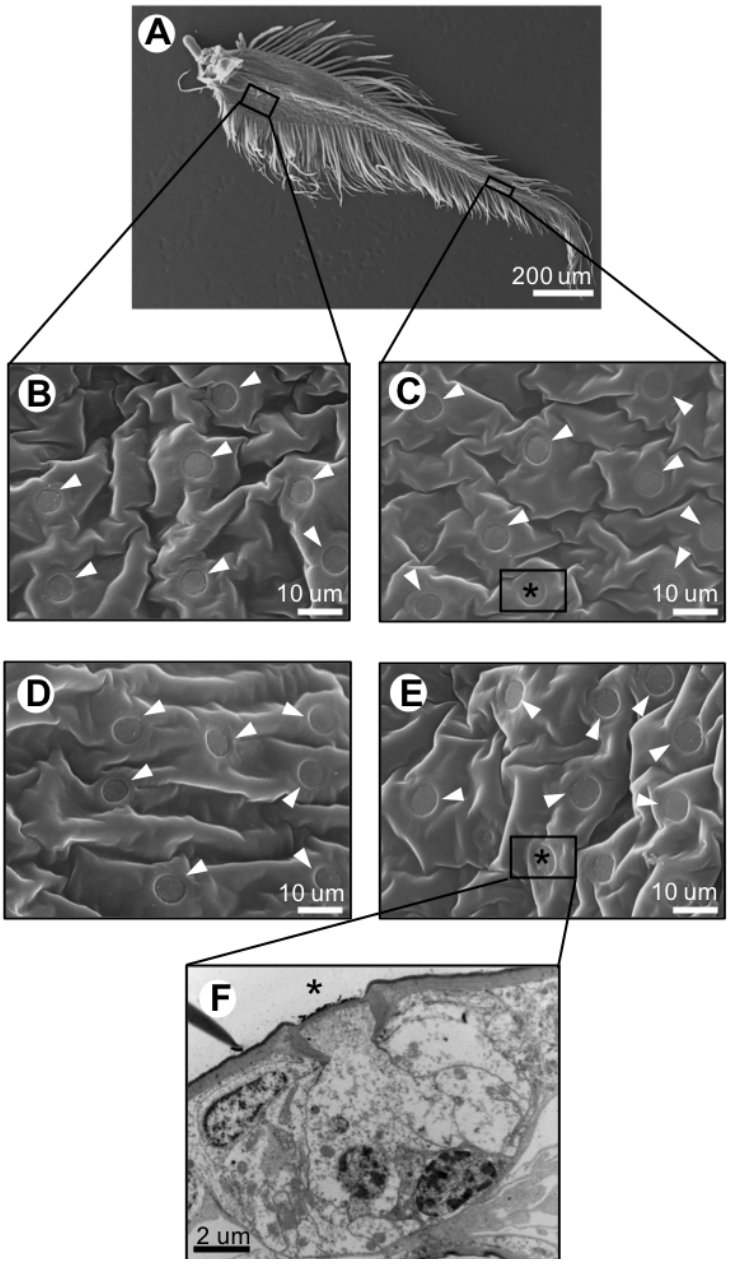

Figure 2-4: Scanning electron microscope (SEM) images of (A) a mayfly (*Hexagenia rigida*) nymph gill filament with magnified representative images taken from the (B) proximal and (C) distal gill filament regions of control animals held in freshwater (FW) as well as the (D) proximal and (E) distal gill filament regions of animals acclimated to salt-contaminated water (SCW). The apical region of tracheal gill ionocytes are indicted by the white arrowheads. In panel (F), a transmission electron micrograph shows a section through the region of the epithelium/cuticle (i.e. apical plate) overlying an ionocyte (as indicated by a white arrowhead in the SEM images). Scale bar in panel (A) = 200 μm, in panels (B-E) = 10 μm and in panel (F) = 2 μm.

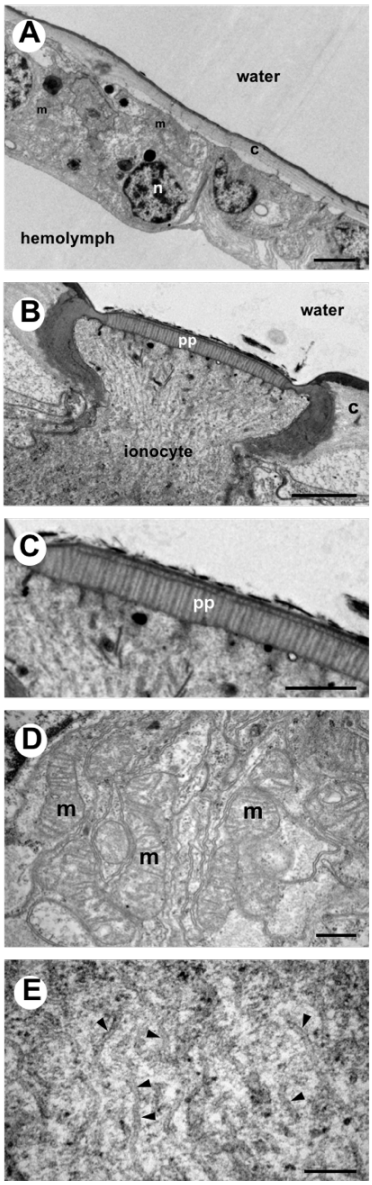

Figure 2-5: Transmission electron microscope (TEM) images of (A) a mayfly (*Hexagenia rigida*) nymph gill filament epithelium and (B-E) different regions of a mayfly gill ionocyte. Panel (A) shows that the gill filament epithelium typically resides underneath a cuticle of comparatively uniform morphology and thickness (i.e. ~ 650 – 800 nm depth). In panel (B), the apical region of a gill epithelium ionocyte can be seen. The morphology of the cuticle is modified in this region (when compared to regular cuticle) and is thinner at ~ 280 nm depth. Panel (C) shows a magnified region of the cuticle overlying the ionocyte [i.e. a magnified image of panel (B)] and reveals what is generally considered to be morphology that reflects a porous condition; hence the term porous plate is often used to describe this area. Panel (D) shows a magnified image of numerous mitochondria that are abundant within tracheal gill ionocytes while panel (E) is a representative image of what appears to be a tubular system (indicated by black arrowheads) within the cell cytoplasm. In this case the structures are in the sub-apical region. Scale bars are as follows: (A) = 2 µm, (B) = 1 µm, (C,D) = 500 nm and (E) = 200 nm. c = cuticle, n = nucleus, m = mitochondrion, and pp = porous plate.

2.4.5 Morphometric analysis of ionocyte surface exposure:

Morphometric measurements of ionocyte exposure were examined by determining individual ionocyte apical area (Fig. 2-6A), ionocyte fractional surface area (i.e. the number of whole or partial ionocytes exposed within a given area) (Fig. 2-6B) and the number of ionocytes exposed per unit tracheal gill area (Fig. 2-6C). These morphometric measurements were determined for both FW and SCW-acclimated animals as well as spatially along the gill (i.e. at the proximal end as well as the distal end). No significant spatial differences or differences between treatments (i.e. FW versus SCW) were found in any of the measured parameters. Ionocyte apical surface area ranged from ~ 7 – 8 μm^2 (Fig. 2-6A), while ionocyte fractional surface area ranged from ~ 3.4 – 3.9 % (Fig. 2-6B). The number of ionocytes exposed at the surface of the gill ranged from ~ 4.5 – 5.0 ionocytes mm^{-2} (Fig. 2-6C).

2.4.6 Ultrastructural observations of gill epithelium septate junctions (SJs):

In the tracheal gill epithelium the morphology of regions of cell-to-cell contact, where occlusion of the intercellular space by SJs would be expected to occur, appeared to substantially differ between nymphs residing in FW (Fig. 2-7A-D) and those acclimated to SCW (Fig. 2-7E – H). More specifically, in FW nymph gills, SJs typically exhibited a convoluted architecture (Fig. 2-7A,C) and occlusion of the intercellular space was typically seen close to the apical surface (Fig. 2-7B,D). In addition, septa spanning the intercellular space in the sub-apical region were comparatively numerous in the tortuous architecture of FW nymph SJs (Fig. 2-7B,D). In contrast, the architecture of SJs in SCW-acclimated nymphs was often more simplified than those often observed in FW nymphs (Fig. 2-7E–H). Furthermore, occlusion of the intercellular space in the sub-apical region was not obvious (Fig. 2-7E-H) and septa spanning the intercellular space were infrequent (Fig. 2-7F,H).

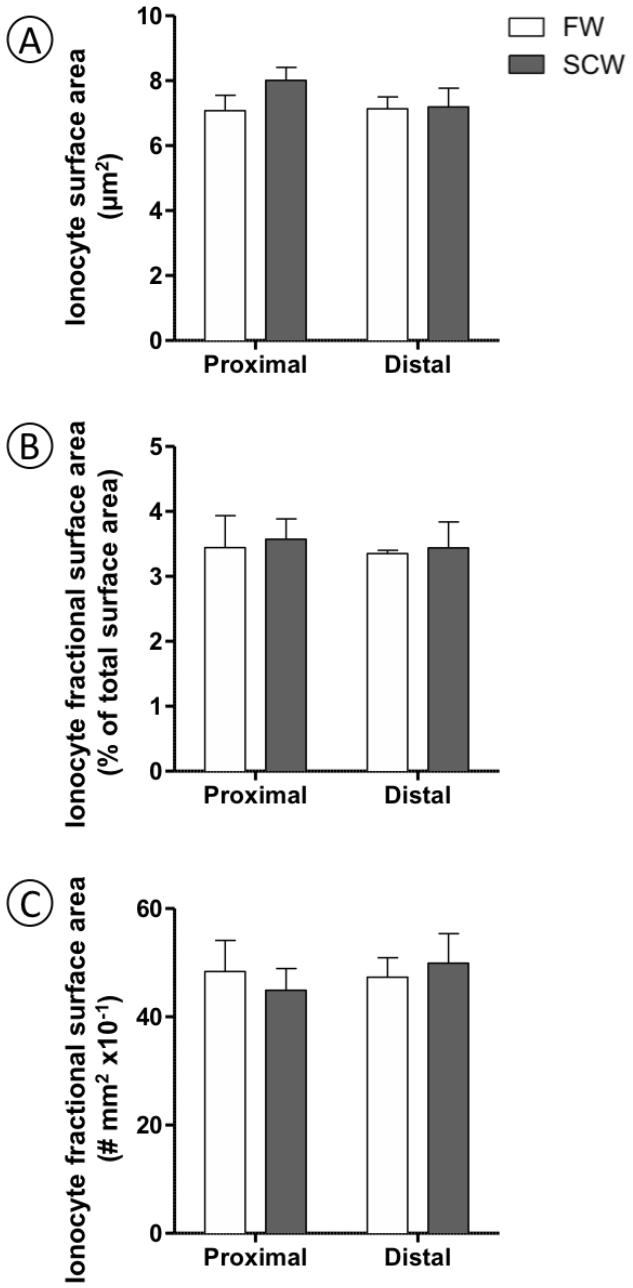

Figure 2-6: Apical surface morphometrics of mayfly (*Hexagenia rigida*) nymph tracheal gill ionocytes in animals residing in freshwater (FW, control) as well as those acclimated to salt-contaminated water (SCW). Morphometric analysis was conducted on ionocytes of the proximal and distal gill epithelium and included (A) ionocyte apical area, (B) ionocyte fractional surface area and (C) the number of ionocytes exposed. Data are expressed as mean values ± s.e.m., n = 5.

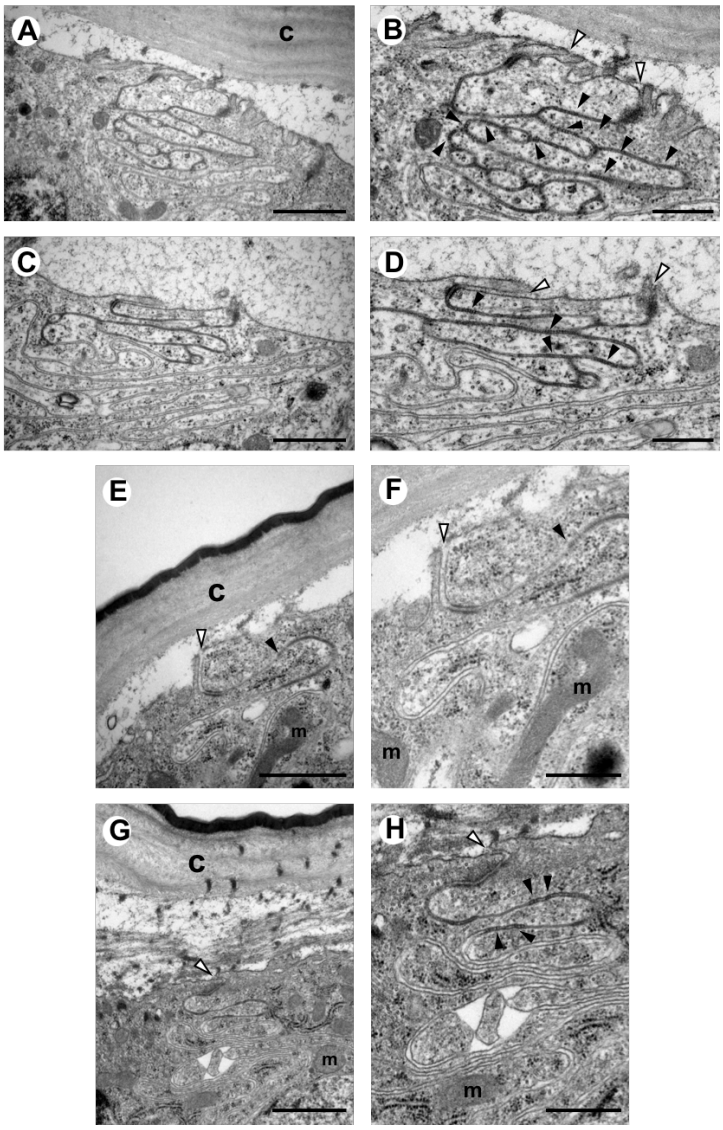

Figure 2-7: Representative transmission electron microscope (TEM) images showing regions of cellular occlusion and septate junctions (SJs) between epithelial cells in the tracheal gill of mayfly (*Hexagenia rigida*) nymphs acclimated to either (A-D) freshwater (FW) or (E-H) salt-contaminated water (SCW). Two representative images of junction regions from FW nymph gills are shown in (A) and (C) and magnified in (B) and (D) respectively. Two representative images of junction regions from SCW-acclimated nymphs are shown in (E) and (G) and are magnified in (F) and (H) respectively. White arrowheads (with a black outline) point to apical-most region of cell-to-cell contact while black arrowheads indicate septa of a SJ spanning the intercellular space in the sub-apical region. Scale bars are as follows: (A, C, E, G) = 1 μm and (B, D, F, H) = 500 nm. c = cuticle and m = mitochondrion.

2.5 Discussion

2.5.1 Overview:

The current study considered the impact of a 7-day SCW exposure on mayfly nymph ionoregulatory homeostasis as well as tracheal gill morphology and physiology. The tracheal gill is an enigmatic organ. This is because it is quite broadly accepted to play a role in mayfly nymph osmoregulation, as first suggested by Wichard and Komnick, (1971), but how it does this is poorly understood. With this in mind, the current study provides further insight into the osmoregulatory physiology of the tracheal gill and in particular how a sublethal concentration of salt contamination (7.25 g/L NaCl) can influence the morphology and physiology of an osmoregulatory epithelium in this organism. The current study demonstrates that exposure to SCW for 7 days significantly influenced systemic salt and water balance as both haemolymph ion levels as well as whole body water content were perturbed. However, common endpoints of ionoregulatory physiology (i.e. ion motive enzyme activities, tracheal gill ionocyte numbers or tracheal gill ionocyte exposure) that might be expected to reflect systemic ion perturbation by changing in response to SCW exposure were not found to have significantly altered. Nevertheless, SIET measurements of Na^+ transport across the gill showed that the gill epithelium had reorganized its function. More specifically, voltage gradients indicated that tracheal gills acquire Na^+ from FW. However, gills taken from animals acclimated to SCW exhibited Na^+ secretion when solute movement was examined in an osmotically clamped solution (i.e. a solution with an osmolality equivalent to SCW). In short, results show that gill function may have changed from being Na^+ absorptive in FW to Na^+ secretory in SCW. This presents somewhat of a paradox given that profound changes in solute movement across an ion transporting epithelium in response to altered environmental ion levels would typically be expected to associate with alterations in endpoints of transcellular ion transport. However, it seems plausible that paracellular solute transport may play a role in Na^+ secretion across SCW nymph gills, either by

utilizing diffusion gradients across/through the paracellular space or via septate junctions (SJs) that may be selectively permeable. In this regard, observations of cell-to-cell occlusion morphology in the sub-apical region of the tracheal gill epithelium, as well as SJ ultrastructure indicate significant alterations in the architecture of the intercellular cleft between gill epithelial cells, which supports the idea that a change in gill epithelium paracellular permeability properties occurs.

2.5.2 Haemolymph ion levels and body water content in *H. rigida* nymphs exposed to SCW:

Haemolymph Na^+, K^+ and Cl^- levels in SCW-acclimated *H. rigida* were significantly elevated and these observations are consistent with previous studies that have reported elevated haemolymph ion levels in FW arthropod larvae following acclimation to salinated FW (Ramsey, 1953; Thornton and Sauer, 1972; Barker and Wilhm, 1982; Jonusaite et al., 2011). We are unaware of any study that has specifically reported alterations in haemolymph ion levels of a mayfly nymph following acclimation to salinated conditions. However, Dowse et al., (2017) examined haemolymph osmolality of a population of *Austrophlebioides pusillus* nymphs residing in salt contaminated water with ion composition resembling that of sea water. Nymphs were exposed to synthetic marine salt water of 1, 2 and 4 g l^{-1}, and at 96 hours 52% of nymphs in 4 g l^{-1} died even though haemolymph osmolality (measured throughout the 96 hour treatment) was no different from controls. On the other hand, Chadwick et al., (2002) examined haemolymph osmolality of a salt-tolerant population of *H. limbata* (a closely related species to *H. rigida* used in this study) that naturally resides within the seasonally saline reaches of the Mobile river in Alabama. From observations of *H. limbata* nymphs experimentally acclimated to brackish water of 5 or 8 ‰ (‰ ~ g l^{-1}) for 7 days, Chadwick et al., (2002) reported no mortality in either salinity as well as no significant elevation in haemolymph osmolality in animals acclimated to 5 ‰. However, haemolymph osmolality of nymphs residing in 8 ‰ for 7 days significantly increased (versus FW controls). Given that these latter experimental conditions closely resembled those of the current study (i.e. 7 days acclimation to 7.25 g l^{-1} NaCl as

compared with 7 days acclimation to 8‰, ~ 8 g l^{-1} brackish water), the results of our study would appear to be consistent with those of Chadwick et al., (2002). Because stenohaline FW arthropods have a limited ability to increase haemolymph osmolality by elevating organic solutes (Griffith, 2016), data suggest that observed changes in haemolymph osmolality (when they occur) may be due to changing haemolymph ion levels, and this will likely occur through a combination of passive water loss or salt uptake from/into the haemocoel as well as altered function of ionoregulatory epithelia. In this regard, an increase in whole body water content was also found in SCW nymphs, and it seems reasonable to consider that a modest shift of water from the extracellular space (i.e. haemolymph) to the intracellular space (where the bulk of water resides in metazoans) in SCW nymphs could cause this, while at the same time contribute to a rise in haemolymph ion levels. Even a modest redistribution of water between fluid compartments would also help nymphs residing in SCW to mitigate dehydration by reducing water loss across externally exposed surfaces.

Interestingly, another mayfly nymph (*Maccaffertium sp.*) showed non-saturable increases in whole body Na^+ uptake rates when acclimated for 5 days in $NaHCO_3$ concentrations as high as ~15 mmol l^{-1}, despite regulating total body Na^+ levels (Scheibener et al., 2016). It was concluded that Na^+ secretion must also have increased under these conditions (Scheibener et al., 2016). This would be a similar strategy as that of the euryhaline osmoconformer *Culex tarsalis*, a mosquito that can inhabit relatively high salt levels (~70% seawater), which was shown to increase Na^+ efflux with increasing salinity to counter increased influx of Na^+ (Patrick et al., 2001). This strategy resulted in attenuation of increases in haemolymph Na^+ in *C. tarsalis* which is apparently similar to what was observed for *Maccaffertium sp.* (Patrick and Bradley, 2000; Scheibener et al., 2016). On the other hand, the increased haemolymph ion levels observed in *H. rigida* in the current study are similar to other stenohaline freshwater insects that are considered to be osmoregulators. For example, both *Culex quinquefasciatus* and *Aedes aegypti* exhibited elevation of haemolymph ion levels with attenuation

of Na^+ uptake rates when held in salt water [≤ 30% seawater] (Patrick et al., 2001; Donini et al., 2007). Although we did not measure Na^+ uptake kinetics in *H. rigida*, we measured Na^+ secretion from the gills of nymphs held in SCW in an osmotically clamped solution, suggesting that *H. rigida* can limit Na^+ uptake as salinity increases. This result is inconsistent with a recent hypothesis (Hypothesis 1) proposed by Kefford (2019) which suggests that mayflies may be more sensitive to salinity compared with other FW animals because of elevated Na^+ uptake rates as salinity increases and the associated energetic cost (Kefford, 2019). It should be noted that the hypotheses proposed by Kefford (2019) are based on a studies by Scheibener et al., (2016) and Buchwalter et al., (2018) which studied nymphs of *Maccaffertium sp.* and *Neocloeon triangulifer* which are not burrowing mayflies and possess gills that are smaller with no fringes compared to the burrowing nymphs of *H. rigida*.

In marine decapods capable of tolerating salinity change, an alkalosis of haemolymph has been associated with acclimation of animals from high to low salinity environments (Henry and Cameron, 1982; Truchot, 1981) and changes in pH over time have been shown to relate closely to increased strong ion difference (SID, i.e. Na^+ - Cl^- as a convenient indicator of overall SID). An alkalosis of the haemolymph in more dilute water compared with salt water, with a corresponding increase in SID has also been shown in the FW mosquito and midge larvae, *A. aegypti* and *C. riparius* (Donini et al., 2007; Jonusaite et al., 2011). Increases in SID in more dilute water was also observed in salt tolerant mosquitoes and caddisflies (Nicolson, 1972; Sutcliffe, 1961). Although these latter studies did not measure haemolymph pH, given the correlation with SID, it may be assumed that these organisms also exhibit haemolymph alkalosis in more dilute water. In contrast to these examples, a modest but significant increase in haemolymph pH was observed in *H. rigida* following exposure to SCW indicating that *H. rigida* suffer haemolymph alkalosis with increased salinity rather than with dilute water. Furthermore, an increase in SID was also observed to occur following acclimation of nymphs to SCW. Therefore, perhaps the relative sensitivity of *H. rigida* nymphs to

salt compared with other organisms is based on their acid base physiology which appears to be different from, at least, the aforementioned organisms which tolerate higher levels of salt. Kefford proposed a hypothesis (Hypothesis 2) where localized alkalization of cells may be caused by increased uptake of Na^+ in ionocytes with a corresponding increase in H^+ secretion (Kefford, 2019). This hypothesis relies on coupling of Na^+ uptake with H^+ secretion which, to the best of our knowledge, has yet to be demonstrated in mayflies. Nevertheless, our observations of a systemic alkalization in *H. rigida* in response to salt suggests that this hypothesis warrants further investigation.

2.5.3 The effect of SCW on activity and immunolocalization of gill NKA and VA:

Following acclimation of euryhaline aquatic organisms to environments that vary in water salt content, common endpoints of functional change in ionoregulatory epithelia are expression or activity measurements of primary active transport enzymes such as NKA and VA as well as changes in the number, size and/or distribution of ionocytes that exhibit an enriched complement of these ion motive enzymes (Evans et al., 2005; Weihrauch et al., 2001; Towle et al,. 2001; Laiz-Carrion et al., 2005). Despite this, we are unaware of any study that has used NKA- or VA-ir to examine gill ionocyte responses of a stenohaline FW arthropod following exposure to salinated water and studies that have examined the activity of these enzymes in the gill of stenohaline FW organisms exposed to salinated water are rare and appear to be restricted to fishes (e.g. Reis-Santos et al., 2008; Woo and Tong, 1982). In the current work, no significant difference was found in the activity of either NKA or VA in the mayfly nymph gill following acclimation to SCW. These observations are in accord with the response of stenohaline FW larval lamprey (Reis-Santos et al., 2008) and snakehead *Ophiocephalus maculatus* (Woo and Tong 1982), both of which displayed no change in gill NKA activity despite exhibiting increased extracellular fluid ion levels following exposure to brackish water ranging from 7 – 10 ‰. In this regard, there would appear to be an across species response of stenohaline FW animal gills to salinated water that may point to a comparatively uniform mechanism

of limited salt water toleration. Nevertheless, we are unaware of any study that has reported how VA activity responds in the gill of a stenohaline FW organism following exposure to salinated water, and while it is generally acknowledged that NKA and VA play a role in ion uptake across the gill epithelium of FW aquatic arthropods, our understanding of how they contribute is based largely on a diadromous species of crab, with limited insight using a hololimnetic species (see Weihrauch et al. 2004).

In agreement with the above, observations of NKA- and VA-ir showed little to no changes in gill ionocyte numbers, size and distribution in *H. rigida* nymphs following acclimation to SCW for 7 days. Much like unaltered *H. rigida* nymph gill enzyme activity in SCW animals, these NKA- and VA-ir observations contrast with what can be observed in the gills of a euryhaline organism (such as a fish) following salinity exposure, where changes in the aforementioned endpoints can be pronounced (Evans et al. 2005). More notable, however, is that the observations in this study also differ from those of Wichard et al. (1973) who reported that a significant decline in gill ionocyte numbers occurred in the gills of the mayfly *C. coloradensis* following exposure to salinated water for 15 days with a measured NaCl concentration of 120 mmol l^{-1} at the end of the 15 day period. This compares well with the current study where levels were 120.7 mmol l^{-1} after the 7 day exposure. However, Wichard et al. (1973) also found that exposure of *C. coloradensis* nymphs to the NaCl treatment water resulted in the death of approximately 52% of experimental animals, and ionocyte numbers were subsequently examined in the survivors. Therefore, to what extent the response reported by Wichard et al., (1973) relates to physiologically relevant morphological alterations is unclear. Finally, to the best of our knowledge, no study has assessed gill chloride cell morphometrics in nymphs exposed to SCW long enough to undergo molting which may or may not be relevant.

2.5.4 Na^+ transport across and ionocyte morphometrics of the tracheal gills of mayfly nymphs residing in FW or SCW:

Na^+ flux was examined spatially along the central axis of the tracheal gill epithelium of nymphs residing in FW as well as those acclimated to SCW. The idea that the tracheal gill epithelium is involved in the acquisition of ions from FW was first introduced by Wichard and Komnick (1971), but direct evidence has only recently been presented (Nowghani et al., 2017). In the latter study, SIET revealed that Na^+ uptake occurred from the proximal to distal end of the gill, and the results of the current study are consistent with these observations. In contrast, Na^+ transport measured across gills taken from SCW-acclimated nymphs showed that these animals may possess the capacity to secrete Na^+. In addition, Na^+ secretion across the gills of SCW animals was significantly greater at the proximal end of the structure than at the distal end. This capacity for secretion did not appear to be reflected by measured changes in ion motive enzyme activity (as noted above) or alterations in ionocyte number as indicated by immunostaining and SEM analysis. In addition, when ionocyte surface exposure morphometrics were examined in both the proximal and distal region of the gill, it was found that neither ionocyte apical area nor ionocyte fractional surface area altered following SCW exposure. Indeed, ionocyte surface ultrastructure remained uniform as well. These observations contrast with reports of gill ionocyte surface ultrastructure and morphometrics of both stenohaline FW fishes and euryhaline fishes, which exhibit significant alterations when animals are exposed to water that varies in salt content (e.g. Kelly et al., 1999; Lee et al., 1996). Furthermore, changes in the morphometrics of fish gill ionocytes are closely tied to alterations in solute uptake in FW (Perry et al., 1992). This would suggest that if there is a transcellular component to Na^+ secretion across the mayfly nymph gill, the reversal in solute transport direction occurs using a reconfiguration of ion transport proteins other than NKA and VA, that utilizes the existing morphology (and number) of

ionocytes. However, another explanation might be that the nymph gills are able to facilitate Na^+ secretion across the paracellular pathway and, at least in part, bypass transcellular ion transport.

2.5.5 Regions of paracellular occlusion in the tracheal gills of mayfly nymphs residing in FW or SCW:

The SJ complex plays a central role in establishing paracellular occlusion barriers in invertebrate epithelia (for review see Jonusaite et al., 2016). Recently, alterations in the molecular physiology and ultrastructure of arthropod SJs in response to environmental change have been linked to changes in the paracellular permeability properties of epithelia in both aquatic and terrestrial arthropods (Jonusaite et al., 2016, 2017a, 2017b; MacMillan et al., 2017). In terms of ultrastructure, MacMillan et al. (2017) reported that SJs of the *Drosophila* midgut epithelium in animals exhibiting low gut permeability were highly convoluted and comparatively richer in septa compared to animals that exhibited a compromised ('leakier') midgut barrier, which were found to possess a simpler SJ morphology. Similar observations were made in mayfly nymph gills, where SJs of animals residing in FW exhibited a more convoluted morphology and comparatively abundant septa when compared to SJs of SCW-acclimated animals. These morphological observations would be consistent with the idea that the gill epithelium of mayfly nymphs acclimated to SCW is leakier than the gill epithelium of FW animals. Furthermore, in addition to architectural changes in the SJ complex that may facilitate Na^+ leak, a Na^+ gradient exists between haemolymph and surrounding water that would favor outwardly directed movement (i.e. Na^+ levels are higher in haemolymph than surrounding SCW).

2.5.6 Conclusion:

In order for aquatic organisms to overcome the challenges presented by a change in environmental salt content, biochemical, morphological and physiological changes must occur. Euryhaline organisms that have evolved to reside in conditions where temporal changes in water salt content are the norm are capable of accomplishing this as a matter of routine. However, the majority

of aquatic organisms live in conditions where even modest changes in water salt content would not be experienced during their lifecycle. In the face of FW salination that occurs as a result of human activity, it is critical to understand the limitations of, and sub-lethal mechanisms that allow stenohaline FW organisms to cope so that we can gain insight into how these animals may be impacted in a broader context. Mayfly nymphs could be considered a salination-sensitive aquatic arthropod because many are stenohaline FW organisms that reside for prolonged periods in finite bodies of FW that (1) are often comparatively small in volume and (2) would not under normal circumstances undergo periodic or long term natural salination. The current work provides a first look at some of the mechanisms that allow the nymph of at least one species of mayfly to cope with sub-lethal levels of FW salt contamination. Although very significant changes in ion transport occur and ionoregulatory homeostasis is disturbed, many typical biochemical and morphological endpoints of ion transport remained unchanged. Moving forward, these observations underscore at least two important points. First is that it is vital to consider the impact of salination on stenohaline FW organisms more broadly. Second, it is imperative to consider the impact of salination from an integrative perspective, in order to make sure that if an absence of change in select endpoints occurs, these are not mistaken for tolerance.

2.6 References

Barker, D. M., & Wilhm, J. (1982). Oxygen Consumption Rates in Sediments and of *Chironomus riparlus* (Meigen) and *Chaoborus punctipennis* (Say) in an Artifically Destratified Lakea. Journal of Freshwater Ecology, 1(6), 607-613.

Barros, R., Isidoro, D., & Aragüés, R. (2012). Three study decades on irrigation performance and salt concentrations and loads in the irrigation return flows of La Violada irrigation district (Spain). Agriculture, Ecosystems & Environment, 151, 44-52.

Beermann, A. J., Elbrecht, V., Karnatz, S., Ma, L., Matthaei, C. D., Piggott, J. J., & Leese, F. (2018). Multiple-stressor effects on stream macroinvertebrate communities: a mesocosm experiment manipulating salinity, fine sediment and flow velocity. Science of the Total Environment, 610, 961-971.

Buchwalter, D., Scheibener, S., Chou, H., Soucek, D., & Elphick, J. (2018). Are sulfate effects in the mayfly *Neocloeon triangulifer* driven by the cost of ion regulation? Philosophical Transactions of the Royal Society B, 374(1764), 20180013.

Cañedo-Argüelles, M., Hawkins, C. P., Kefford, B. J., Schäfer, R. B., Dyack, B. J., Brucet, S., ... & Coring, E. (2016). Saving freshwater from salts. Science, 351(6276), 914-916.

Chadwick, M. A., & Feminella, J. W. (2001). Influence of salinity and temperature on the growth and production of a freshwater mayfly in the Lower Mobile River, Alabama. Limnology and Oceanography, 46(3), 532-542.

Chadwick, M. A., Hunter, H., Feminella, J. W., & Henry, R. P. (2002). Salt and water balance in *Hexagenia limbata* (Ephemeroptera: Ephemeridae) when exposed to brackish water. Florida Entomologist, 85(4), 650-652.

Chasiotis, H., & Kelly, S. P. (2008). Occludin immunolocalization and protein expression in goldfish. Journal of Experimental Biology, 211(10), 1524-1534.

Cormier, S. M., Suter, G. W., Zheng, L., & Pond, G. J. (2013). Assessing causation of the extirpation of stream macroinvertebrates by a mixture of ions. Environmental Toxicology and Chemistry, 32(2), 277-287.

Corsi, S. R., Graczyk, D. J., Geis, S. W., Booth, N. L., & Richards, K. D. (2010). A fresh look at road salt: aquatic toxicity and water-quality impacts on local, regional, and national scales. Environmental Science & Technology, 44(19), 7376-7382.

Donini, A., Gaidhu, M. P., Strasberg, D. R., & O'Donnell, M. J. (2007). Changing salinity induces alterations in hemolymph ion concentrations and Na^+ and Cl^- transport kinetics of the anal papillae in the larval mosquito, *Aedes aegypti*. Journal of Experimental Biology, 210(6), 983-992.

Donini, A., & O'Donnell, M. J. (2005). Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. Journal of Experimental Biology, 208(4), 603-610.

Dowse, R., Palmer, C. G., Hills, K., Torpy, F., & Kefford, B. J. (2017). The mayfly nymph *Austrophlebioides pusillus* Harker defies common osmoregulatory assumptions. Royal Society Open Science, 4(1), 160520.

Fay, L., & Shi, X. (2012). Environmental impacts of chemicals for snow and ice control: state of the knowledge. Water, Air, & Soil Pollution, 223(5), 2751-2770.

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiological reviews, 85(1), 97-177.

Griffith, M. B. (2017). Toxicological perspective on the osmoregulation and ionoregulation physiology of major ions by freshwater animals: teleost fish, Crustacea, aquatic insects, and Mollusca. Environmental Toxicology and Chemistry, 36(3), 576-600.

Yong, H. S., & Yule, C. M. (2004). Freshwater invertebrates of the Malaysian region. Akademi Sains Malaysia.

Henry, R. P., & Cameron, J. N. (1982). Acid-base balance in *Callinectes sapidus* during acclimation from high to low salinity. Journal of Experimental Biology, 101(1), 255-264.

Howard, K. W., & Maier, H. (2007). Road de-icing salt as a potential constraint on urban growth in the Greater Toronto Area, Canada. Journal of Contaminant Hydrology, 91(1-2), 146-170.

Jonusaite, S., Kelly, S. P., & Donini, A. (2011). The physiological response of larval *Chironomus riparius* (Meigen) to abrupt brackish water exposure. Journal of Comparative Physiology B, 181(3), 343-352.

Jonusaite, S., Kelly, S. P., & Donini, A. (2013). Tissue-specific ionomotive enzyme activity and K^+ reabsorption reveal the rectum as an important ionoregulatory organ in larval *Chironomus riparius* exposed to varying salinity. Journal of Experimental Biology, 216(19), 3637-3648.

Jonusaite, S., Donini, A., & Kelly, S. P. (2016). Occluding junctions of invertebrate epithelia. Journal of Comparative Physiology B, 186(1), 17-43.

Jonusaite, S., Donini, A., & Kelly, S. P. (2017). Salinity alters snakeskin and mesh transcript abundance and permeability in midgut and Malpighian tubules of larval mosquito, *Aedes aegypti*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 205, 58-67.

Jonusaite, S., Kelly, S. P., & Donini, A. (2017). Identification of the septate junction protein gliotactin in the mosquito *Aedes aegypti*: evidence for a role in increased paracellular permeability in larvae. Journal of Experimental Biology, 220(13), 2354-2363.

Kaushal, S. S., Groffman, P. M., Likens, G. E., Belt, K. T., Stack, W. P., Kelly, V. R., ... & Fisher, G. T. (2005). Increased salinization of fresh water in the northeastern United States. Proceedings of the National Academy of Sciences, 102(38), 13517-13520.

Kaushal, S. S., Duan, S., Doody, T. R., Haq, S., Smith, R. M., Johnson, T. A. N., ... & Wood, K. L. (2017). Human-accelerated weathering increases salinization, major ions, and alkalinization in fresh water across land use. Applied Geochemistry, 83, 121-135.

Kefford, B. J. (2018). Why are mayflies (Ephemeroptera) lost following small increases in salinity? Three conceptual osmophysiological hypotheses. Philosophical Transactions of the Royal Society B, 374(1764), 20180021.

Kefford, B. J., Marchant, R., Schäfer, R. B., Metzeling, L., Dunlop, J. E., Choy, S. C., & Goonan, P. (2011). The definition of species richness used by species sensitivity distributions approximates observed effects of salinity on stream macroinvertebrates. Environmental Pollution, 159(1), 302-310.

Kefford, B. J., Hickey, G. L., Gasith, A., Ben-David, E., Dunlop, J. E., Palmer, C. G., ... & Piscart, C. (2012). Global scale variation in the salinity sensitivity of riverine macroinvertebrates: Eastern Australia, France, Israel and South Africa. PLoS ONE, 7(5), e35224.

Kelly, S. P., Chow, I. N., & Woo, N. Y. (1999). Haloplasticity of black seabream (*Mylio macrocephalus*): hypersaline to freshwater acclimation. Journal of Experimental Zoology, 283(3), 226-241.

Kelly, V. R., Lovett, G. M., Weathers, K. C., Findlay, S. E., Strayer, D. L., Burns, D. J., & Likens, G. E. (2007). Long-term sodium chloride retention in a rural watershed: legacy effects of road salt on streamwater concentration. Environmental Science & Technology, 42(2), 410-415.

Kolosov, D., Piermarini, P. M., & O'Donnell, M. J. (2018). Malpighian tubules of *Trichoplusia ni*: recycling ions via gap junctions and switching between secretion and reabsorption of Na^+ and K^+ in the distal ileac plexus. Journal of Experimental Biology, 221(5), jeb172296.

Laiz-Carrión, R., Guerreiro, P. M., Fuentes, J., Canario, A. V., Martín Del Río, M. P., & Mancera, J. M. (2005). Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus auratus*. Journal of Experimental Zoology Part A: Comparative Experimental Biology, 303(7), 563-576.

Lee, T. H., Hwang, P. P., & Feng, S. H. (1996). Morphological studies of gill and mitochondria-rich cells in the stenohaline cyprinid teleosts, *Cyprinus carpio* and *Carassius auratus*, adapted to various hypotonic environments. Zoological Studies, 35, 272-278.

Likens, G. E., & Buso, D. C. (2010). Salinization of Mirror Lake by road salt. Water, Air, and Soil Pollution, 205(1-4), 205.

MacMillan, H. A., Yerushalmi, G. Y., Jonusaite, S., Kelly, S. P., & Donini, A. (2017). Thermal acclimation mitigates cold-induced paracellular leak from the Drosophila gut. Scientific Reports, 7(1), 8807.

Müller, B., & Gächter, R. (2012). Increasing chloride concentrations in Lake Constance: characterization of sources and estimation of loads. Aquatic Sciences, 74(1), 101-112.

Nicolson, S. W. (1972). Osmoregulation in larvae of the New Zealand salt-water mosquito *Opifex fuscus* Hutton. Journal of Entomology Series A, General Entomology, 47(1), 101-108.

Nicolson, S. W. (1972). Osmoregulation in larvae of the New Zealand salt-water mosquito *Opifex fuscus* Hutton. Journal of Entomology Series A, General Entomology, 47(1), 101-108.

Nowghani, F., Jonusaite, S., Watson-Leung, T., Donini, A., & Kelly, S. P. (2017). Strategies of ionoregulation in the freshwater nymph of the mayfly *Hexagenia rigida*. Journal of Experimental Biology, 220(21), 3997-4006.

Panno, S. V., Hackley, K. C., Hwang, H. H., Greenberg, S. E., Krapac, I. G., Landsberger, S., & O'kelly, D. J. (2006). Characterization and identification of Na-Cl sources in ground water. Groundwater, 44(2), 176-187.

Patrick, M. L., Gonzalez, R. J., & Bradley, T. J. (2001). Sodium and chloride regulation in freshwater and osmoconforming larvae of Culex mosquitoes. Journal of Experimental Biology, 204(19), 3345-3354.

Patrick, M. L., & Bradley, T. J. (2000). The physiology of salinity tolerance in larvae of two species of Culex mosquitoes: the role of compatible solutes. Journal of Experimental Biology, 203(4), 821-830.

Perry, S., Goss, G., & Laurent, P. (1992). The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts. Canadian Journal of Zoology, 70(9), 1775-1786.

Pond, G. (2010). Patterns of Ephemeroptera taxa loss in Appalachian headwater streams (Kentucky, USA). Hydrobiologia, 641(1), 185-201.

Pond, G., Passmore, M. E., Borsuk, F. A., Reynolds, L., & Rose, C. J. (2008). Downstream effects of mountaintop coal mining: comparing biological conditions using family-and genus-level macroinvertebrate bioassessment tools. Journal of the North American Benthological Society, 27(3), 717-737.

Reis-Santos, P., McCormick, S. D., & Wilson, J. M. (2008). Ionoregulatory changes during metamorphosis and salinity exposure of juvenile sea lamprey (*Petromyzon marinus L.*). Journal of Experimental Biology, 211(6), 978-988.

Saouter, E., Le Menn, R., Boudou, A., & Ribeyre, F. (1991). Structural and ultrastructural analysis of gills and gut of *Hexagenia rigida* nymphs (Ephemeroptera) in relation to contamination mechanisms. Tissue and Cell, 23(6), 929-938.

Scheibener, S. A., Richardi, V. S., & Buchwalter, D. B. (2016). Comparative sodium transport patterns provide clues for understanding salinity and metal responses in aquatic insects. Aquatic Toxicology, 171, 20-29.

Sutcliffe, D. W. (1961). Studies on salt and water balance in caddis larvae (Trichoptera): I. Osmotic and ionic regulation of body fluids in *Limnephilus affinis* Curtis. Journal of Experimental Biology, 38(3), 501-519.

Sutcliffe, D. W. (1962). The Composition of Haemolymth in Aquatic Insects. Journal of Experimental Biology, 39(3), 325-344.

Swinton, M. W., Eichler, L. W., & Boylen, C. W. (2015). Road salt application differentially threatens water resources in Lake George, New York. Lake and Reservoir Management, 31(1), 20-30.

Thornton, K. W., & Sauer, J. R. (1972). Physiological effects of NaCl on *Chironomus attenuatus* (Diptera: Chironomidae). Annals of the Entomological Society of America, 65(4), 872-875.

Towle, D. W., Paulsen, R. S., Weihrauch, D., Kordylewski, M., Salvador, C., Lignot, J. H., & Spanings-Pierrot, C. (2001). Na^+/K^+-ATPase in gills of the blue crab Callinectes sapidus: cDNA sequencing and salinity-related expression of α-subunit mRNA and protein. Journal of Experimental Biology, 204(22), 4005-4012.

Truchot, J. P. (1981). The effect of water salinity and acid-base state on the blood acid-base balance in the euryhaline crab, *Carcinus maenas* (L.). Comparative Biochemistry and Physiology Part A: Physiology, 68(4), 555-561.

Thunqvist, E. L. (2004). Regional increase of mean chloride concentration in water due to the application of deicing salt. Science of the Total Environment, 325(1-3), 29-37.

Weihrauch, D., Morris, S., & Towle, D. W. (2004). Ammonia excretion in aquatic and terrestrial crabs. Journal of Experimental Biology, 207(26), 4491-4504.

Weihrauch, D., Ziegler, A., Siebers, D., & Towle, D. (2001). Molecular characterization of V-type H^+-ATPase (B-subunit) in gills of euryhaline crabs and its physiological role in osmoregulatory ion uptake. Journal of Experimental Biology, 204(1), 25-37.

Wichard, W., Tsui, P., & Komnick, H. (1973). Effect of different salinities on the coniform chloride cells of mayfly larvae. Journal of Insect Physiology, 19(9), 1825-1835.

Wichard, W., & Komnick, H. (1971). Electron Microscopial and Histochemical Evidence of Chloride Cells in Tracheal Gills of Mayfly Larvae. Cytobiologie, 3(2), 215-228.

Williams, D., Williams, N., & Cao, Y. (2000). Road salt contamination of groundwater in a major metropolitan area and development of a biological index to monitor its impact. Water Research, 34(1), 127-138.

Wingfield, C. A. (1939). The function of the gills of mayfly nymphs from different habitats. Journal of Experimental Biology, 16(3), 363-373.

Woo, N. Y. S., & Tong, W. C. M. (1982). Salinity adaptation in the snakehead, *Ophiocephalus maculatus* Lacépède: changes in oxygen consumption, branchial Na^+-K^+-ATPase and body composition. Journal of Fish Biology, 20(1), 11-19.

Zall, D. M., Fisher, D., & Garner, M. Q. (1956). Photometric determination of chlorides in water. Analytical Chemistry, 28(11), 1665-1668.

CHAPTER 3:

FLUID AND ION SECRETION BY THE EXCRETORY SYSTEM OF MAYFLY NYMPHS (*HEXAGENIA RIGIDA*): EFFECTS OF SECOND MESSENGERS AND SALINITY

3.1 Summary

Salt contamination of freshwater (FW) from human activities (e.g. mining, agriculture and winter de-icing) is a global concern. FW dwelling mayfly nymphs appear to be particularly sensitive to salt contamination but very little is known about their ion/osmoregulatory response to this environmental insult. This study found that exposing nymphs of the mayfly (*Hexagenia rigida*) to salt contaminated water (SCW; 7 days in FW containing 7.25 g/L NaCl) resulted in a significant decrease in K^+ reabsorption at the Malpighian tubules (MTs), an essential organ comprising the excretory system. To isolate the function of the MTs, Ramsay secretion assays were utilized to measure fluid secretion rates of MTs from FW and SCW nymphs in response to various diuretic factors. Neither the FW nor SCW MTs secreted fluid unless stimulated. cAMP resulted in elevated secretion rates in both FW and SCW tubules, cGMP completely inhibited fluid secretion in the SCW tubules while thapsigargen promoted fluid secretion in SCW tubules. Interestingly, in SCW a significant decline in Na^+-K^+-ATPase and V-type H^+-ATPase activities in the MT, may suggest the role of cGMP in response to increased salinity. This study provides a first look at how salt contamination of FW can impact the normal transport processes of a mayfly nymph renal system.

3.2 Introduction

Heavy metals and pesticides are prominent freshwater (FW) contaminants that are widely studied by toxicologists, however only recently FW salinization has also become a major cause of water degradation (Bitton et al., 1994; Castillo et al., 2006; Kaushal et al., 2017). There are many different sources of salt; however, in the northern hemisphere, the salinization of FW ecosystems by de-icing road salt is a significant and growing problem that has been linked to increased urbanization (Kaushal et al. 2005). The high use of road salt draws a lot of concern for the health of FW ecosystems, including those of FW bodies as large as the great lakes of Ontario, because salinization of FW by road salt de-icing runoff can have a major impact on the physiology and fitness of FW

organisms (Ontario's Great Lakes Strategy 2016 Progress Report). Road salt is primarily composed of NaCl and once in contact with water, will separate to sodium and chloride ions. Chloride ions have been increasing in the Great Lakes since the mid 1990s and pose a serious threat to drinking water quality as well as aquatic life (Water Quality in Ontario 2014 Report). Although multiple stressors, such as seasonal change in abiotic factors (*e.g*. temperature) as well as agriculture runoff, may influence chloride levels and/or create hypoxic conditions, road salt is a significant contributing factor (Kaushal et al., 2005). Thus, in order to asses this situation regulators must be fully informed of the consequences of salt contamination on FW ecosystems and the organisms that reside within them. In this regard, it is imperative for us to understand how the physiology of FW organisms that would not normally encounter elevated environmental salt levels is influenced by FW salinization.

All FW organisms are faced with the challenge of maintaining salt and water balance and as a result they depend on osmoregulation to support homeostasis (Willmer, 2006). Using physiological and/or behavioral mechanisms, osmoregulation allows an organism to keep a constant internal concentration of ions, sometimes even when faced with changing environmental conditions (Willmer, 2006). For a FW organism, the challenge is to retain or acquire salts that are lost from the body by passive diffusion to surroundings that are more dilute than blood (haemolymph). Osmoregulation of organisms, such as aquatic insect larvae, occurs across specialized epithelia of osmoregulatory organs and these maintain ion gradients between fluid compartments (Bradley, 1987). Haemolymph homeostasis in aquatic insect larvae is largely regulated across epithelia of the alimentary canal (Dow 1987; O'Donnell 2008). The alimentary canal is subdivided into functionally distinct regions such as the foregut, midgut, Malpighian tubules (MTs) and hindgut. The midgut is the site of mineral, water and nutrient absorption, and is also responsible for the acid-base balance (Dow 1987; Linser et al., 2009). The hindgut, composed of ileum and rectum, work with the MTs to complete the excretory system. The MTs produce a primary urine through active secretion of ions and the subsequent osmotic

influx of water from the haemolymph into their lumen (Jonusaite et al., 2013). The composition of the primary urine is adjusted through the actions of the rectum prior to excretion. The rectum contributes to the regulation of haemolymph homeostasis by selectively reabsorbing ions which results in the production of a dilute urine (Phillips, 1981). In FW insect larvae, rectal ion reabsorption helps to reduce ion loss as well as eliminate excess water, thus maintaining tissue water levels. Having a key role in the osmoregulation of aquatic animals are plasma membrane energizers V-type H^+-ATPase (VA) and Na^+/K^+-ATPase (NKA) (Del Duca et al., 2011). In epithelial cells of osmoregulatory organs, these enzymes coordinate to drive solute transport. The activity of VA and NKA can be altered depending on the physiological stressors an organism is facing (Harvey, 1998). Targeting mostly the MTs, neuroendocrine factors regulate the tubules by stimulating or inhibiting fluid secretion (Zadeh-Tahmasebi et al., 2016). The biogenic amine 5- hydroxytryptamine (5-HT) has been shown to act through the second messenger adenosine 3',5'-cyclic monophosphate (cAMP) to stimulate fluid secretion (Clark and Bradley, 1996). Whereas the second messenger cyclic guanosine monophosphate (cGMP) has been shown to both stimulate and inhibit fluid secretion depending on the activating agent and the model organism (Massaro et al., 2004; Pollock et al., 2004). In this regard, there is no information on the hormonal regulation of mayfly nymph tubules, making it more difficult to have a holistic understanding of their physiology.

Mayfly (Ephemeroptera) nymphs are recognized as significant bioindicators of FW quality (Brittain, 1982) and are prominent insects in northern FW ecosystems. Therefore, insight into mayfly osmoregulatory physiology and the influence of salt contamination on the ability of these organisms to maintain salt and water balance will contribute significantly to a broader understanding of the deleterious effects of FW salinization in N. America. The current study sought to address a significant knowledge gap in our understanding of mayfly physiology using *Hexagenia rigida* as a model for examination of the effects of FW salinization. More specifically, it can be hypothesized that the

burrowing and extended FW *H. rigida* nymph form will be significantly impacted by FW salinization. To consider this idea further, we examined the water and ion balance mechanisms carried out by the Malpighian tubules of *H. rigida* nymphs.

3.3 Materials and Methods

3.3.1 Animals:

H. rigida nymphs (16-35 mm in length) were collected from a culture maintained by the Ontario Ministry of the Environment, Conservation and Parks (OMECP, Etobicoke, ON, Canada). See section 2.3.1 for details.

3.3.2 Salt contaminated water (SCW) exposure and measurement of osmolality:

Treatment conditions were adapted from Chadwick et al., 2002, in which *H. limbata* nymphs (a closely related species to *H. rigida*, that often coexist) were shown to regulate haemolymph osmolality at salinities < 8ppt for 7 days. In order to avoid lethality, *H. rigida* nymphs were exposed to SCW, which was established by dissolving 7.25 g l^{-1} of NaCl (BioShop Canada Inc.) in dechlorinated municipal tap water. Size matched nymphs were transferred to 2 l containers containing Lake Erie sediment (approx. 7.6 cm deep) with either FW (control) or SCW for a period of 7 days, while salinity levels were regulated by measuring Cl^- according to Zall et al. (1956). At the end of the 7-day exposure samples of FW and SCW media were collected from the treatment tanks and centrifuged at 10 000 rpm for 1 minute to separate the sediment from the water. The osmolality of the supernatant was determined by freezing point depression using an Osmette II osmometer (Precision System, Inc., Natick, MA). Standard solutions were purchased from Precision Systems.

3.3.3 Haemolymph osmolality:

Following the removal from experimental water, nymphs were rinsed with double distilled water to remove residual salts and blotted with Kimwipe™ to remove residual moisture. Nymphs were immobilized on a Sylgard® (Dow Corning, Mississauga, ON, Canada) filled glass petri dish

base, after which the cuticle was punctured at the base of a leg to allow pooling of haemolymph. Haemolymph sample was then collected using a micropipette and stored in microcentrifuge tubes at -80 °C. Samples were thawed before osmolality was measured using the same technique described for water above.

3.3.4 Na^+-K^+-ATPase (NKA) and V-type H^+ -ATPase (VA) activity measurements:

For enzyme analysis, the alimentary canal was dissected out and the hindgut as well as all five MT branches (Fig. 3-1) were isolated under physiological saline (composition in mmol l^{-1}: 5 KCl, 74 NaCl, 1 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 8.6 Hepes, 20 glucose, 10 glutamine, pH 7.0). Due to small tissue size, tissues from 3 nymphs were pooled into microcentrifuge tubes to achieve n of 1, after which they were flash frozen in liquid nitrogen. During dissection, tubes were held on ice. Samples were stored at −80 °C until use. NKA and VA activities were measured according to methods outlined in detail by (McCormick, 1993) and modified by Jonusaite et al. (2011). Enzyme inhibitor concentrations for NKA and VA were 5 mmol l^{-1} of ouabain and 1 μmol l^{-1} of bafilomycin respectively, in accordance with those reported by Nowghani et al. (2017).

3.3.5 Immunohistochemical localization of NKA and VA:

To carry out wholemount immunohistochemistry, the Malpighian tubules were isolated from the alimentary canal in physiological saline and processed according to Jonusaite et al., 2013. Immunohistochemical localization of NKA and VA was carried out as mentioned in section 2.3.6. Images were captured using an Olympus BX-51 laser-scanning confocal microscope. All images were assembled using ImageJ software (ImageJ ver.1.51J8, National Institutes of Health, USA).

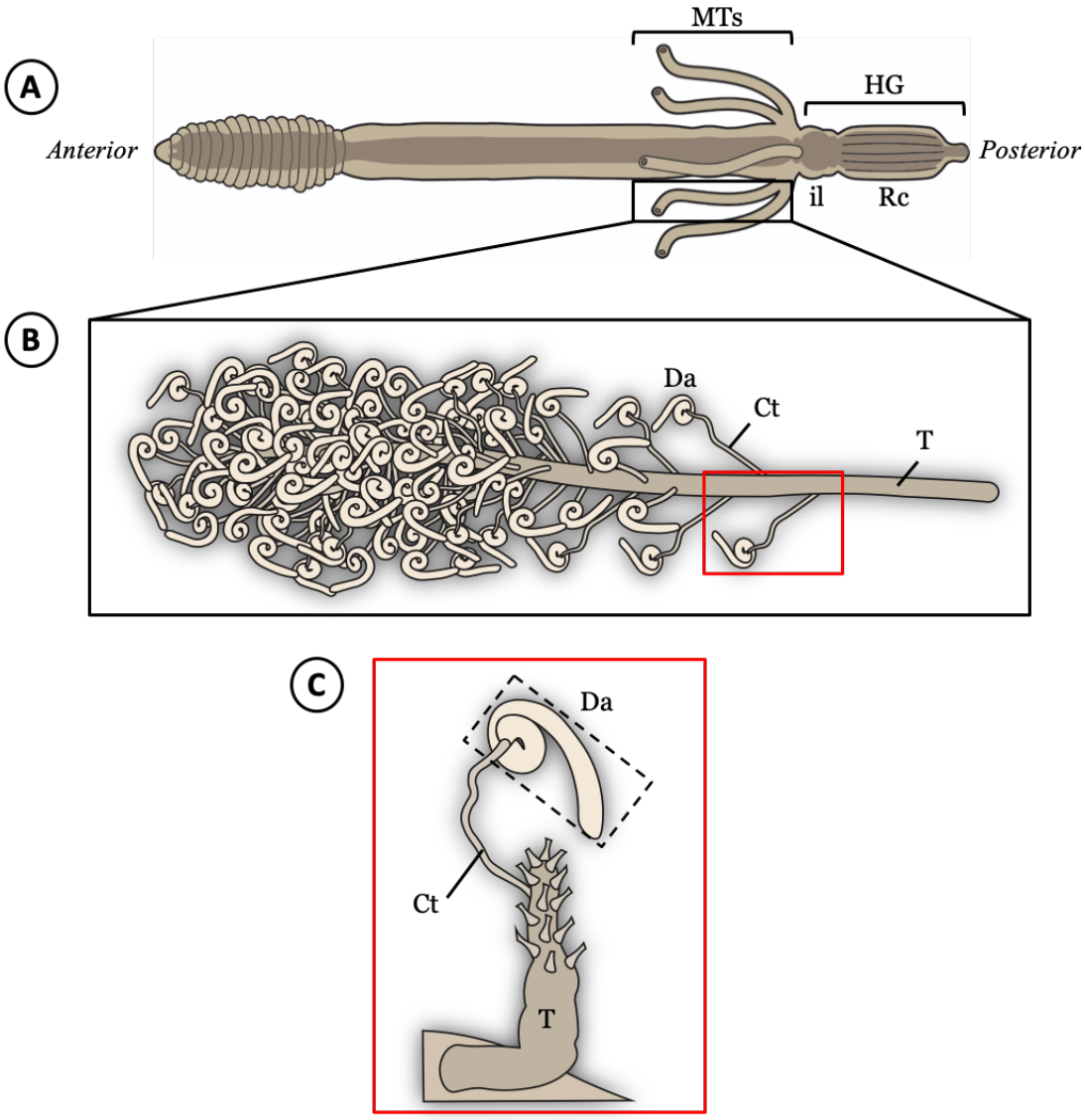

Figure 3-1: Mayfly (*Hexagenia rigida*) nymph Malpighian tubule morphology. (A) Diagram of alimentary canal of *H. rigida* nymphs depicting the Malpighian tubules (MTs) and the hindgut (HG), which is comprised of the ilium (il) and rectum (Rc). (B) one of the five identical mayfly nymph MTs and its branching and tortuous organization. (C) Magnified region of the MT indicated by the red box, with (T) representing the trunk, (Ct) the conducting tube and (Da) the distal apparatus.

3.3.6 K^+ flux along the MTs and rectum:

Scanning Ion-selective Electrode Technique (SIET) measurement of K^+ voltage gradient was used to determine flux rate of this ion along the MTs and rectum. Voltage gradients were recorded along the length of tubules allowing for spatial differentiation of the discrete regions of the MTs as previously described by Nowghani et al., 2017. The SIET system and protocol utilized in this study is described in detail elsewhere (Donini and O'Donnell, 2005; Kolosov et al., 2018). The entire alimentary canal was isolated under physiological saline and placed on a petri dish, with the tubules stretched out perpendicular to the digestive tract. For both FW and SCW nymphs the petri dish was filled with physiological saline (composition as reported above). When calibrated in 1 and 10 mmol l^{-1} solutions of KCl and N-methyl-D-glucamine (NMDG) (for total of 137.3 $mmol^{-1}$), the K^+ ISME slope (mV) for a 10-fold change in ion concentration for the FW and SCW bath was (means ± s.e.m.); (60.08 ±1.2, N=6) for the MTs and (56.3 ± 1.8, N=5) for the rectum.

3.3.7 Calculation of K^+ flux rates from SIET-measured voltage gradients:

The voltage gradient readings which were collected using ASET 2.0 software were converted to concentration gradients using the following equation 2-3 (section 2.3.8). After obtaining the concentration gradients, a flux value for the corresponding gradients was derived using Fick's law of diffusion, as presented by equation 2-4 (section 2.3.8).

3.3.8 Fluid secretion assay:

To determine secretion rates, a modified Ramsay secretion assay (Ramsay, 1954) was performed on isolated *H. rigida* nymph tubules. Two tubule branches were isolated from the base from each nymph under physiological saline. Tubules were then transferred to a sylgard-lined petri dish containing 20 µl bathing droplets submerged beneath hydrated mineral oil to prevent evaporation. The proximal end of the tubule was removed from the bath and anchored in the mineral oil by a minute pin, which allowed the secreted fluid from the tubules to pool into a droplet. To

measure the basal secretion rate, the tubules were placed in a physiological saline bath. To expose the tubules to serotonin, serotonin hydrochloride (5-hydroxytryptamine, 5-HT) (Sigma-Aldrich, Oakville, ON, Canada) was dissolved in ultrapure water to make a 1 mmol l^{-1} stock and further diluted in physiological saline to achieve a 10^{-5} mol l^{-1} concentration, which was used as a bath. Additionally, a dose response relationship was determined for both adenosine 3',5'-cyclic monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Membrane permeable 8-bromo cAMP (Sigma-Aldrich, Oakville, ON, Canada) was diluted in physiological saline to concentrations of 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} mol l^{-1}. Membrane permeable 8-bromo cGMP was dissolved in 100 mM NaOH at a concentration of 10^{-2} mol l^{-1} and diluted in saline for final concentrations of 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} mol l^{-1}. Lastly, thapsigargin was dissolved in dimethyl sulfoxide (DMSO) and diluted in saline for final concentration of 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} mol l^{-1}. Once placed in the corresponding bathing solution, the MTs were allowed to secrete for a maximum period of 60 minutes. Secreted fluid droplets that accumulated at the proximal end of the tubule were then collected for further analysis. The diameter of MT secreted fluid droplets was measured, and the volume of the droplets calculated using the following equation:

Equation 3-1: Volume = $\frac{4}{3}\pi\left(\frac{1}{2}\ diameter\right)^2$

Fluid secretion rates were calculated by dividing volume by the time it took to secrete the fluid droplets. The following procedure was done for MTs of FW and SCW nymphs.

3.3.9 Measurement of [Na^+] and [K^+] in the secreted fluid:

Cation concentrations of secreted fluid were determined using ISME with the following ionophore cocktails (Fluka, Buchs, Switzerland) and back-fill solutions (in parentheses): K^+ ionophore I cocktail B (100 mmol l^{-1} KCl) and Na^+ ionophore II cocktail A (100 mmol l^{-1} NaCl), after which the tips of the ISMEs were coated with polyvinylchloride. The ISMEs were calibrated in the following solutions: K^+ (1.5 mmol l^{-1} KCl/298.5 mmol l^{-1} NaCl and 15 mmol l^{-1} KCl/285 mmol l^{-1}

NaCl; 5 mmol l^{-1} KCl/295 mmol l^{-1} NaCl and 50 mmol l^{-1} KCl/250 mmol l^{-1} NaCl; 10 mmol l^{-1} KCl/280 mmol l^{-1} NaCl and 100 mmol l^{-1} KCl/200 mmol l^{-1} NaCl); Na^+ (5 mmol l^{-1} NaCl/295 mmol l^{-1} LiCl and 50 mmol l^{-1} NaCl/250 mmol l^{-1} LiCl; 30 mmol l^{-1} NaCl/270 mmol l^{-1} LiCl and 300 mmol l^{-1} NaCl). The ISME slopes (mV) for a 10-fold change in ion concentration were (54.0 ± 0.87, N=6) for K^+, (55.6 ± 0.61, N=5) for Na^+.

An ML165 pH Amp connected to a PowerLab 4/30 (ADInstruments, Colorado Springs, CO, USA) data acquisition system was used to measure voltage readings and LabChart 6 Pro software (ADInstruments) was used for analysis. Ion activities were calculated using the following equation 2-1 (section 2.3.3), as described by Donini et al. (2007). The concentration of Na^+ and K^+ ions were then measured in the secreted fluid droplet with ion-selective microelectrodes (ISME, see next section) and calculated with the following equation:

Equation 3-2: $[Ion] = [C] \times 10^{\Delta V/m}$

Where [C] is the concentration of the lowest calibration; ΔV is the difference in voltage between the lowest calibration solution and the sample recorded by ISME. Lastly, m is the difference in voltage between the two calibration solutions, representing the slope of the electrode.

3.3.10 Statistics:

All statistical tests used as the level of significance, unless otherwise $P < 0.05$ stated. Osmolality data was analyzed using unpaired t-test. The effect of rearing salinity and secretagogues on each of the five parameters measured was also analyzed using unpaired t-test. Data presented in figures 3-5G and 3-8C were analyzed using a two-way ANOVA. All data was analyzed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA).

3.4 Results

3.4.1 Treatment water and haemolymph osmolality:

The measured osmolality of the SCW tank was (mean ± s.e.m) 252.6 ± 1.8 mOsm Kg^{-1}, significantly higher than the FW control tank at 29.25 ± 2.5 mOsm Kg^{-1}. As a result, the exposed nymphs acclimated to the new high salinity environment (Fig. 3-2) and adjusted their haemolymph osmolality to 292.2 ± 2.9 mOsm Kg^{-1}, N=10 relative to the control baseline (FW) at 236.75 ± 11.3 mOsm Kg^{-1}.

3.4.2 Activity and Immunolocalization of NKA and VA:

As shown in figure 3-3A, NKA activity was significantly decreased in the MTs of nymphs acclimated to SCW (3.70 ± 0.38 µmol ADP mg^{-1} protein h^{-1}) relative to the FW control (6.27 ± 0.59 µmol ADP mg^{-1} protein h^{-1}). In the FW nymphs VA activity was 1.97 ± 0.30 µmol ADP mg^{-1} protein h^{-1}, in the SCW acclimated nymphs however, no VA activity was detected (Fig. 3-3B). In the hindgut (HG), both NKA and VA activity showed a pattern towards decrease in response to the SCW, however, these changes were not significant (Fig. 3-3A,B). Immunohistochemical examination of the tortuous morphology of the structurally distinct regions of the MTs as depicted in figure 3-3G, revealed basolateral NKA and cytosolic VA immunoreactivity (Fig. 3-3C-F), with visibly less VA localization in the distal regions of the SCW exposed tubules (Fig. 3-3C,D).

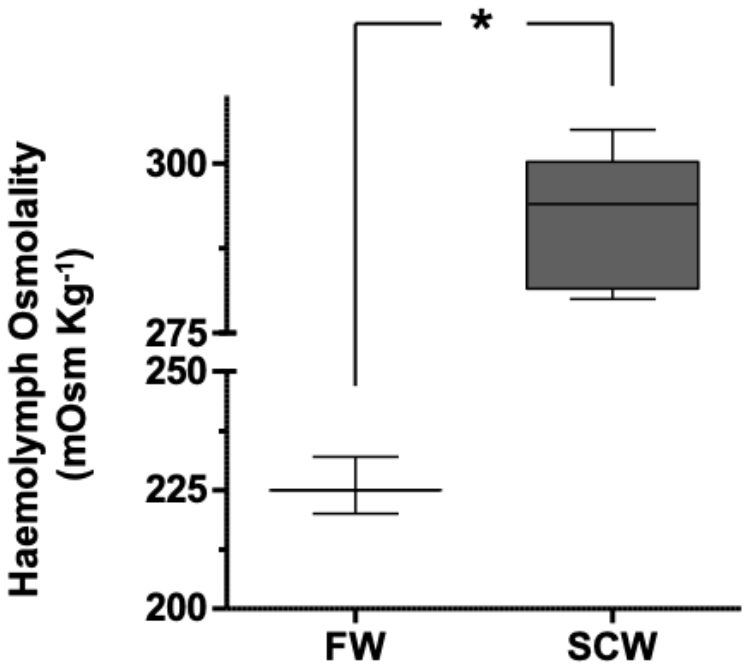

Figure 3-2: The effects of a 7-day exposure to salt-contaminated water (SCW) on haemolymph osmolality of *Hexagenia ridiga* nymphs. An asterisk denotes a statistically significant difference (t-test, $P<0.05$) between freshwater control (FW) and SCW exposed nymphs.

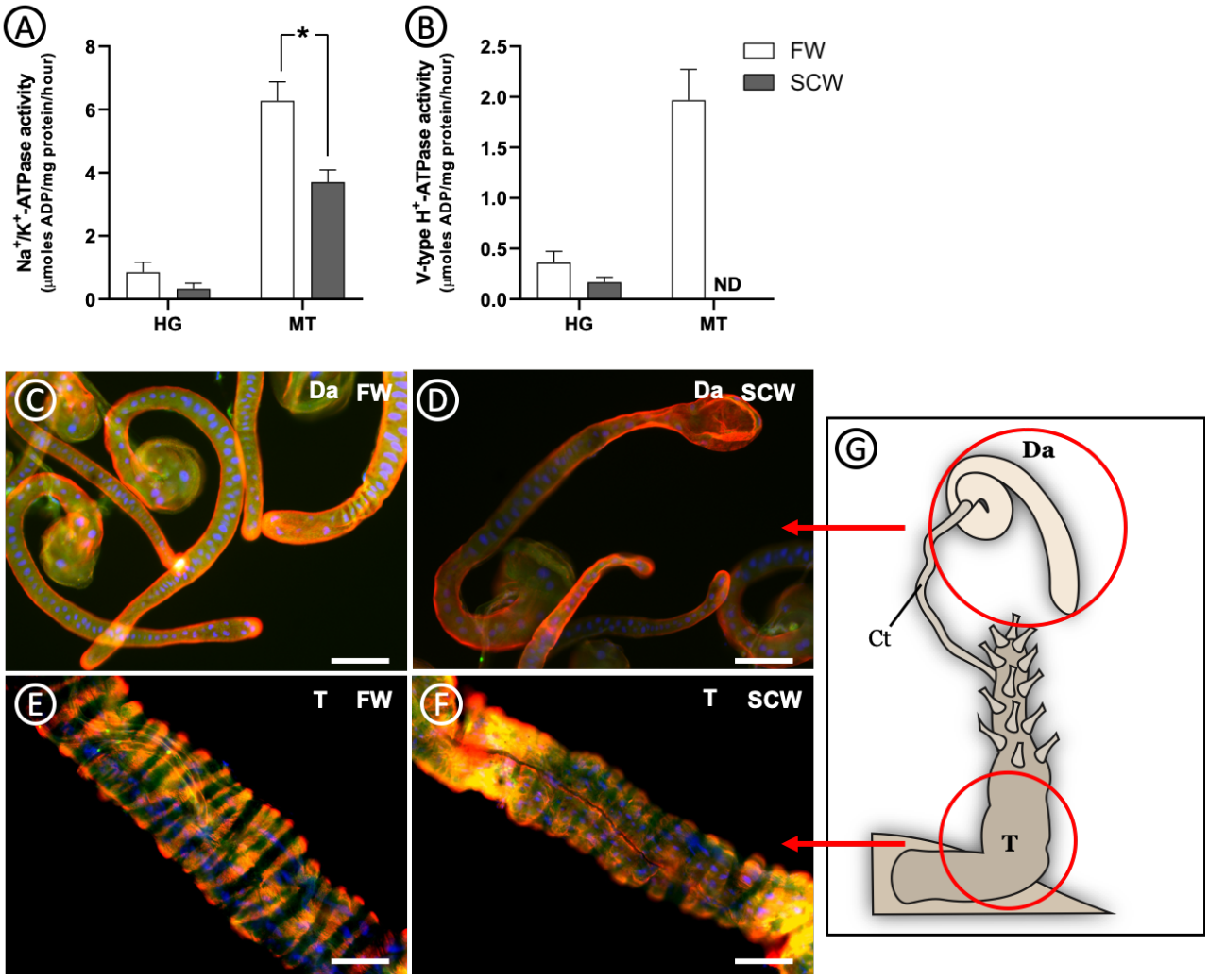

Figure 3-3: The activity of (A) Na^+-K^+-ATPase (NKA) and (B)V-type H^+-ATPase (VA) in the Malpighian tubules (MTs) and hindgut (HG) of *Hexagenia rigida* nymphs held in freshwater (FW) or exposed to salt-contaminated water (SCW) for 7 days. (C,D) immunoreactivity of NKA (red) and VA (green) demonstrate basolateral staining for NKA and apical or cytosolic staining for VA in the distal apparatus of the tubules. NKA and VA staining along the trunk of (E) FW and (F) SCW MTs reveal a unique cellular ultrastructure. (G) simplistic demonstration of *H. rigida* MTs, with letters denoting various tubule regions such as (T) trunk; (Da) distal apparatus and (Ct) conducting tube. The red circles denote regions of the MTs which have been immunohistochemically stained. An asterisk denotes a statistically significant difference (t-test, $P<0.05$) between FW and SCW animals. Scale = 50 µm. (ND) not detected.

3.4.3 K^+ transport along the MTs:

In *H. rigida* nymphs each nymph possesses five MT trunks originating from the gut (see Fig. 3-1 for illustration). Each trunk further branches into morphologically distinct regions called the distal apparatus (Fig. 3-4A). This apparatus begins with the conducting tube, which leads into a coiled-spiral region that is continuous with a blind-ended finger-like projection (Fig. 3-4B). K^+ transport was measured spatially along the distal apparatus and revealed a K^+ efflux (from lumen to bath) in both FW and SCW acclimated nymphs. K^+ measured at the blind-end (Ed.) of the tubules differed significantly between the two treatments (FW: 88.58 ± 14 pmol $cm^{-2}s^{-1}$, N=24) with the SCW tubules displaying significantly lower K^+ reabsorption rates (SCW: 31.93 ± 4.7 pmol $cm^{-2}s^{-1}$, N=7) (Fig. 3-5A,B). The following region, the rod-like extension (Rd.) was subdivided into five regions to enforce simplicity and four out of the five regions displayed significantly lower K^+ reabsorption rates in response to the SCW, with the biggest difference being in sub-region one (R1) (FW: 172.5 ±45 pmol $cm^{-2}s^{-1}$, N=38; SCW: 31.86 ± 2.1 pmol $cm^{-2}s^{-1}$, N=10) (Fig. 3-5C). Additionally, the K^+ reabsorption measured along the spiral region (Spr.) was 62.37 ± 4.6 pmol $cm^{-2}s^{-1}$, N=60 for the FW control and less than half of this value for SCW exposed nymphs (28.24 ± 3.7 pmol $cm^{-2}s^{-1}$, N=27) (Fig. 3-5E,F). Lastly, the K^+ reabsorption rates were measured along the three regions; Ed., Rd., and Spr in both FW and SCW nymphs (Fig. 3-5G). Flux rates measured along the Rd. region were summed up in the FW and SCW treatments (Fig. 3-5D) and compared to the other regions in figure 3-5G. Following a similar trend, K^+ flux measurements along the rectum, revealed a significant decrease in K^+ reabsorption rate in response to the SCWE (FW: 280.8± 27 pmol $cm^{-2}s^{-1}$, N=14; SCW: 152.7± 15 pmol $cm^{-2}s^{-1}$, N=19) (Fig. 3-6).

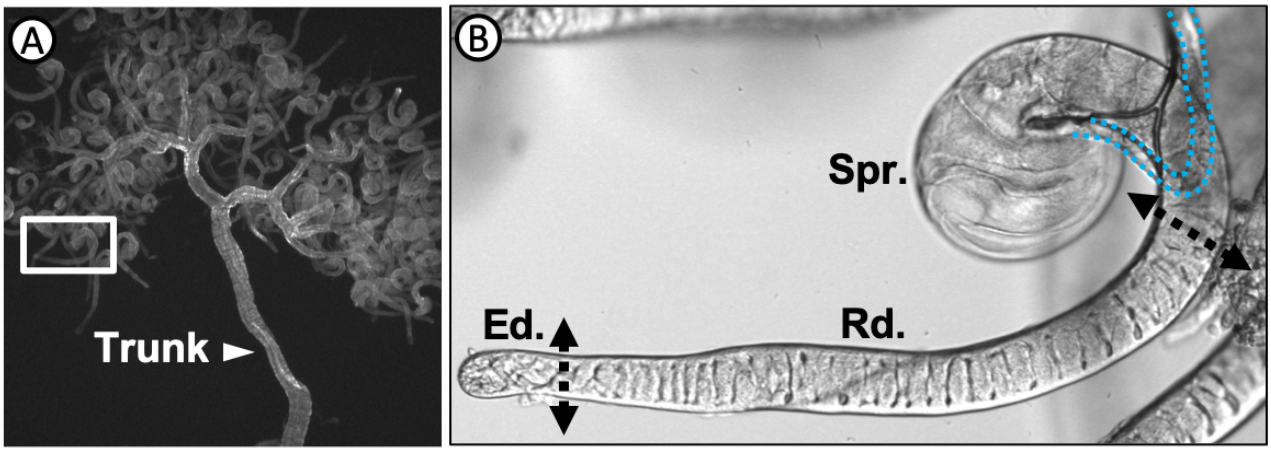

Figure 3-4: Five trunks protrude from the junction between the posterior midgut and the hindgut in *Hexagenia rigida* nymphs, with two trunks being the thickest. (A) one of the two trunks with the white arrow pointing to the proximal end from the gut and white box highlighting the most distal tubule apparatus. (B) the highlighted distal apparatus is composed of a conducting tube (outlined by the dotted blue line) to the spiral (Spr) region, which is continuous with the rod-like (Rd) extension with the blind end (Ed).

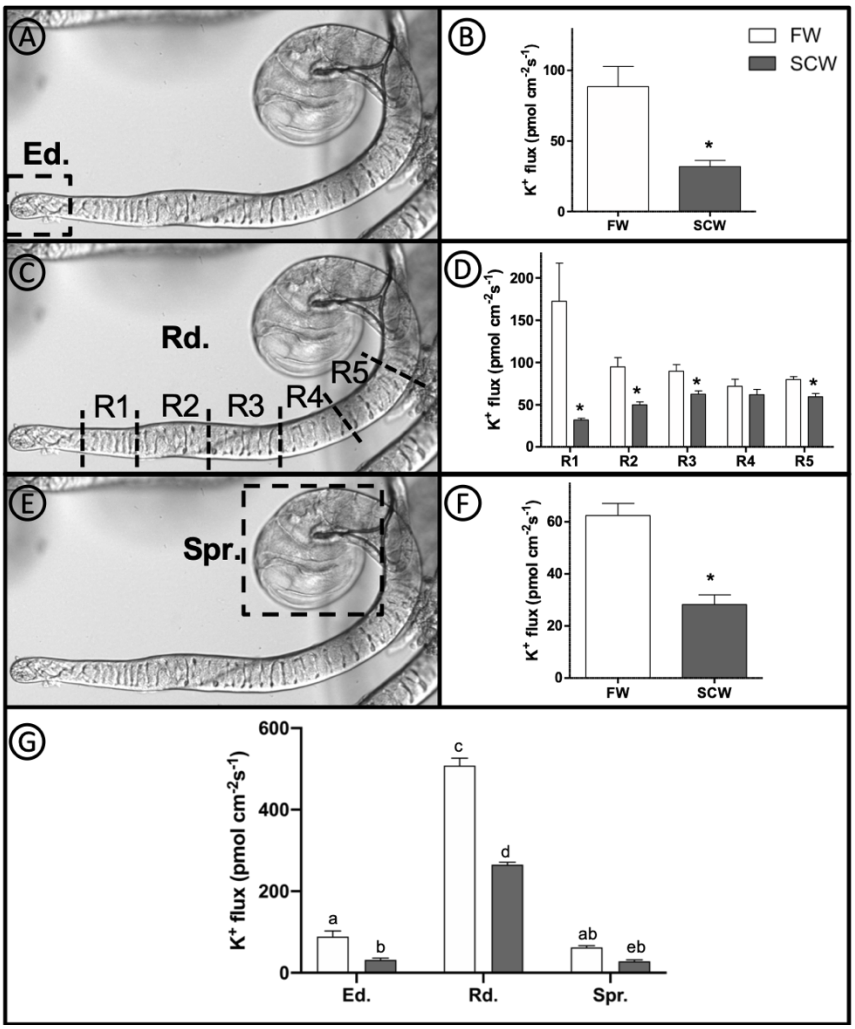

Figure 3-5: Scanning ion-selective electrode technique (SIET) measurement of K^+ flux (B,D,F) across the distal tubule apparatus of *Hexagenia rigida* nymphs held in freshwater (FW) or exposed to salt-contaminated water (SCW). (A,E) the black dotted box indicates the measured region and the black dotted lines (C) subdivide the rod-like extension into five sections for simplicity and regionalized measurements. An asterisk denotes a statistically significant difference (t-test, $P<0.05$) between FW and SCW animals. (G) the measured K^+ flux is compared between the three regions (B,D,F), with the bars from panel D being summed up. Two way-ANOVA depicting significant difference between the different treatments.

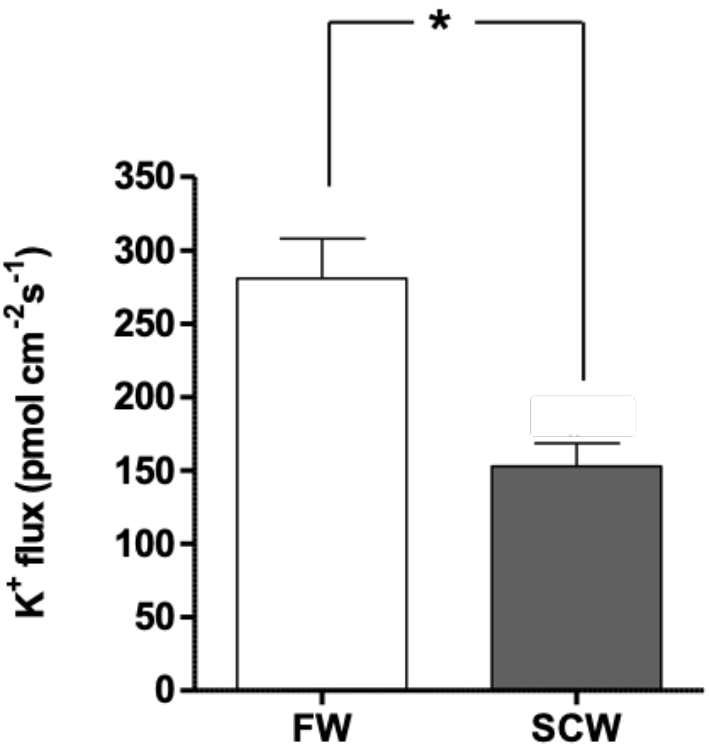

Figure 3-6: Scanning ion-selective electrode technique measurement of K^+ flux across the rectum of mayfly nymph (*Hexagenia rigida*) held in freshwater (FW) or exposed to salt-contaminated water (SCW). All data are expressed as mean values ± SEM; n = 15-16. An asterisk denotes a statistically significant difference (t-test, $P<0.05$) between FW and SCW animals.

3.4.4 The Effects of environmental salinity on secretagogue imposed fluid secretion:

In vitro, the MTs of *H. rigida* did not secrete without stimulation. Thus, 10^{-3} mol l^{-1} of 5-HT was applied to the tubules to stimulate secretion, however, no secretion was observed. Next, second messengers, cAMP and cGMP were applied at various doses and resulted in the dose dependent secretion of tubules. Additionally, Ca^{2+} ATPase inhibitor, thapsigargin was applied also at varying concentrations to establish a dose response curve (3-7A,C-E). At 10^{-3} mol l^{-1} cAMP imposed the highest fluid secretion rate in both FW and SCW tubules (Fig. 3-7A), with the SCW being significantly higher (FW: 197.13 ± 37 nl min^{-1}, N=4; SCW: 294.52 ± 42 nl min^{-1}, N=6). The changes in fluid secretion may be imposed by the significant increase in K^+ concentration seen in the SCW acclimated tubule fluid (FW: 7.15 ± 0.55 mmol l^{-1}, N=7; SCW: 32.79 ± 3.9 mmol l^{-1}, N=5) (Fig. 3-7B). The application of cGMP only stimulated fluid secretion in FW tubules, since the SCW tubules remained unstimulated (Fig. 3-7C). The concentration of cGMP and fluid secretion rate had an inverse relationship, with the lowest fluid secretion rate being at the highest cGMP concentration. In the secreted fluid, Na^+ was the dominant cation (36.55 ± 2.8 mmol l^{-1}, N=4), similar to the cAMP mediated secretion in FW tubules. In contrast, the application of thapsigargin only stimulated SCW fluid secretion at its lowest and highest concentrations (Fig. 3-7E). Applied at its highest concentration, thapsigargin mediated fluid secretion rate was 392.2 ± 42 nl min^{-1}, N=4. Similar to the pattern seen in cGMP-aided secretion at 10^{-3} mol l^{-1}, at the same concentration, thapsigargin displays a similar pattern in that Na^+ is the dominant cation at 213.6 ± 12 mmol l^{-1}, N=8 (Fig. 3-7F). It is noteworthy to notice the significantly higher Na^+ and K^+ levels in the secreted fluid in response to thapsigargin compared to the other secretagogues applied. For instance, Na^+ concentrations as a result of thapsigargin secretion is ~ 6 fold higher than both cAMP or cGMP aided secretion. Similarly, K^+ concentrations in the thapsigargin aided secretion is 9 folds higher than K^+ concentrations in the cGMP secreted fluid and 2 fold higher than the cAMP aided secretion.

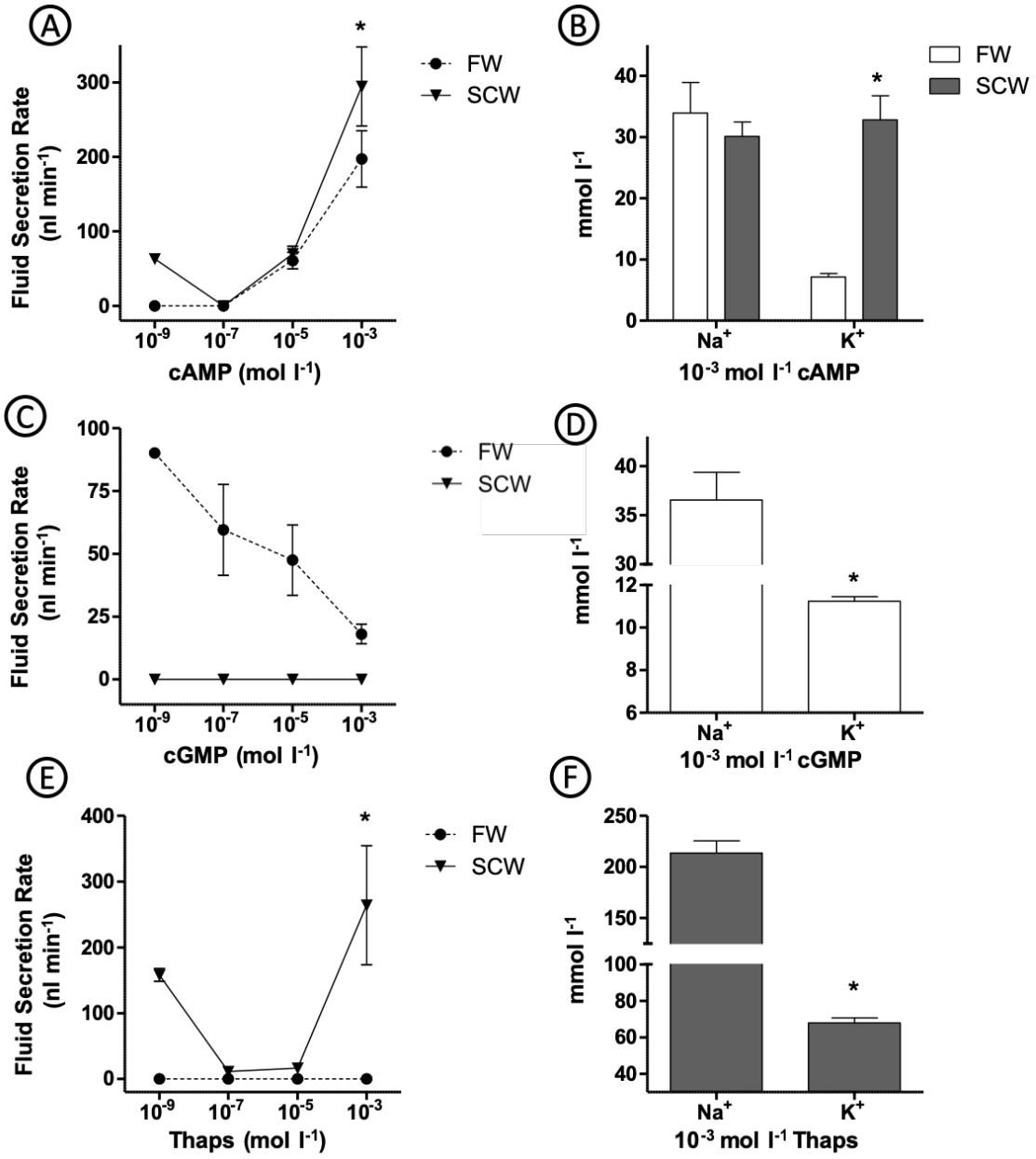

Figure 3-7: (A) The effects of salt-contaminated water (SCW) on cAMP, cGMP or thapsigargin (Thaps)-mediated fluid secretion rate of the Malpighian tubules of *Hexagenia rigida.* The tubules did not secrete unless stimulated. (B) The ionic strength of Na^+ and K^+ ions in the tubules of (B) freshwater (FW) and SCW exposed nymphs, (D) FW kept nymphs and (E) SCW exposed nymphs in the presence of 10^{-3} mol l^{-1} cAMP, cGMP or Thaps. An asterisk denotes a statistically significant difference (t-test, $P<0.05$) between FW and SCW animals.

Since the magnitude of cGMP stimulation of MT fluid secretion in FW tubules had an inverse relationship with dose cGMP was competed with cAMP to examine if there was any inhibitory activity of cGMP. For this, cAMP and cGMP were competed at 10^{-3} mol l^{-1} (Fig. 3-8). When applied together the FW MTs secrete at 355.1 ± 48 nl min^{-1}, N=4 and the SCW exposed nymph tubules secrete at 464.9 ± 70 nl min^{-1}, N=5, both of which are higher than the fluid secretion rates of the second messengers applied independently (Fig. 3-8C).

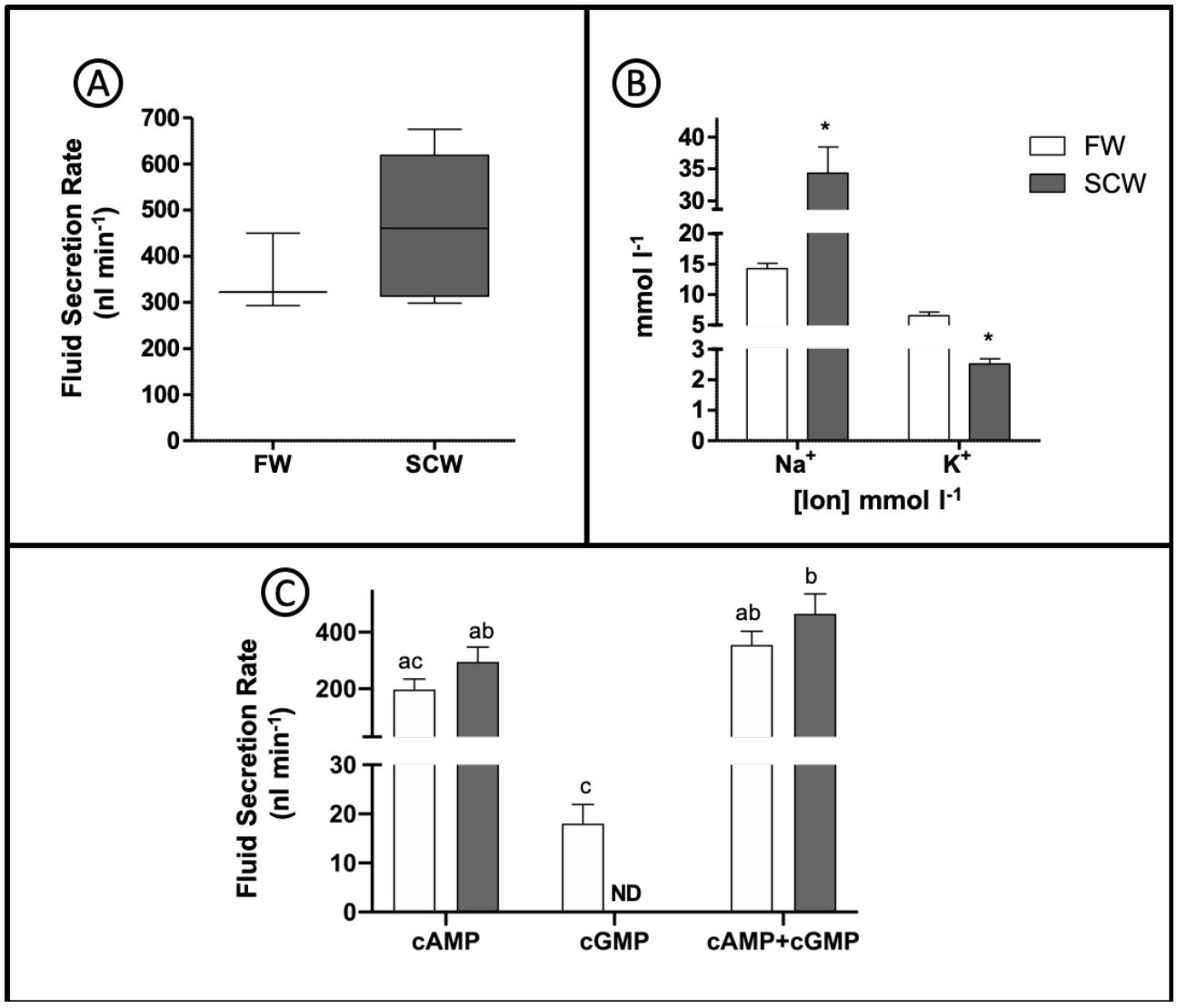

Figure 3-8: (A) the rate of fluid secretion of freshwater (FW) and salt-contaminated water (SCW) exposed *Hexagenia rigida* tubules in the presence of both cAMP and cGMP at 10^{-3} mol l^{-1}. (B) the ionic strength of Na^+ and K^+ ions in the secreted fluid in both FW and SCW tubules in the presence of cAMP and cGMP. An asterisk denotes a statistically significant difference (t-test, $P<0.05$) between FW and SCW animals. (C) FW and SCW fluid secretion rates compared between cAMP-exposed, cGMP-exposed as well as cAMP and cGMP-exposed tubules. Data are expressed as mean values ± SEM. Letters denote significant differences in fluid secretion rates based on a Two-way ANOVA.

3.5 Discussion

3.5.1 Overview:

This study considered the impact of a 7-day SCW exposure on mayfly nymph ionoregulation through the study of their excretory system, the MTs and rectum. As suggested by Nowghani et al., 2019, we can speculate that the increase seen in *H. rigida* haemolymph osmolality is driven by the changing haemolymph ion levels, since stenohaline insects have a limited ability to elevate organic solutes (Griffith, 2017). We can further confirm the suggestion made by Nowghani et al., 2019 that an increase in haemolymph osmolality and ion levels is driven by salt uptake from/into the haemocoel, occurring simultaneously with changes in the function of ionoregulatory epithelia, such as the MTs and the rectum. The MTs and the rectum contribute to the partial regulation by reducing their K^+ reabsorption rate, thus suggesting that other osmoregulatory organs such as the tracheal gills or the midgut maybe contributing to the increased levels of haemolymph K^+ and so the MTs adjust their transport rates in order to prevent K^+ accumulation. To gain a better understanding on the overall contribution of the MTs to achieving this new homeostatic state, the fluid secretion mechanism of the tubules was examined. Over the years, many studies have demonstrated the short-term modulation of fluid secretion in insect MTs by circulating neurohormones and intracellular second messengers (Petzel et al., 1987; Donini et al., 2006; Sajadi et al., 2018). To test that, we noticed in the absence of hormonal factors, alterations in the ambient salinity did not impose changes nor trigger fluid secretion in *H. rigida* tubules. However, we show that ambient salinity has profound effects on fluid secretion in second messenger mediated- secretion.

3.5.2 The effects of SCW on haemolymph osmolality:

Most FW animals are hyper-regulators, maintaining their haemolymph at higher concentrations than the external environment. Thus, using up energy to actively pump ions against the concentration gradient as well as excreting excess water (Kefford, 2019). Given that FW insects

are strict osmoregulators, in a case where external salinity causes the external osmolality to surpass the isosmotic point, the animal may begin to osmoconform and adjust their haemolymph osmolality to match that of the environment (Kefford, 2019). A similar trend can be seen with the *H. rigida* nymphs, where in the freshwater environment, the haemolymph osmolality is well above that of the FW environment, however after chronic exposure to the SCW, the nymphs significantly elevated their haemolymph osmolality to be slightly above that of the environment. It can be speculated that the observed increase in haemolymph osmolality is in part, if not all, due to elevations in major osmolyte levels such as Na^+, K^+ and Cl^- as shown by Nowghani et al., 2019. The latter study also went on to show a significant increase in total body water content, ruling out the possibility of dehydration, implying that the nymphs actively conform to the new environment. Given that Ephemeroptera are the most sensitive to environmental salinity relative to other FW insects such as mosquito larvae, it can be suggested that Ephemeroptera are limited in their ability to osmoconform and are pushed to their survival threshold. Although, the topic of osmoregulation is thought to be very well understood (Dowse et al., 2017), the physiology of FW insects and in particular mayfly nymphs have been very poorly studied.

3.5.3 The effects of SCW on ATPase activity and localization:

Comprising the excretory system, the MTs and the hindgut largely drive haemolymph homeostasis. MT epithelia has been shown to mediate internal homeostasis through ion secretion (movement of ions from the haemolymph into the tubule lumen) and ion reabsorption (movement of ions from the lumen into the haemolymph) (Jonusaite et al., 2013). A homeostatic state is only reached when the rectum (a region of the hindgut) selectively absorbs ions, water and nutrients from the tubule secreted fluid (Bradley 1994). This mechanism is driven by the expression or activity of primary active transport enzymes, NKA and VA, which provide common endpoints of functional changes across not only the tubules and the rectum but other ionoregulatory epithelia as well. In the

current study we showed significant alterations in NKA and VA activity in the MTs in response to the SCW, which to the best of our knowledge have not been reported in other FW insects. For example, a study conducted on midge *Chironomus riparius* larvae reported no changes in the active machinery of the excretory system (Jonusaite et al., 2013). However, they went on to explain that their findings do not preclude changes in ultrastructural organization of the epithelia or secondary ion transport, which maybe driving the change in ion transport (Jonusaite et al., 2013). The same can be speculated for the *H. rigida* rectum, since alterations in the environmental salinity did not trigger alterations in ATPase activity, while altering ion transport (see next section). In this case, the passive ion movement may be a target for coping with SCW, altering the modulation of paracellular permeability via septate junctions. Similar suggestions have been made by Nowghani et al., 2019, since changes in the septate junction organization we reported in *H. rigida* tracheal gills. As for the MTs, the current dogma holds that VA is the dominant force driving tubule activity (Weng et al., 2003), however, we have found that in the *H. rigida* tubules, NKA seems to be the driving force given the greater activity and immunoreactivity with respect to VA. In response to the SCW, NKA activity significantly decreased, which was followed by changes in K^+ reabsorption rates along the tubules (see next section). Dominating the apical membrane conductance, VA activity is crucial for proper tubule function, however, in the following study we find that in response to the SCW, no VA activity was detected in the *H. rigida* tubules. Studies which have inhibited VA in the tubules have found that fluid secretion has also been inhibited (Maddrell and O'Donnell, 1992). This however does not seem to be the case here, since the tubules continue to function. To get a more complete understanding of the tubules, we also looked at the ATPase activity in the hindgut of *H. rigida* and was surprised to find that neither NKA nor VA activity were altered in response to the SCW. Given the lack of research in this area in mayfly nymphs, it is difficult to speculate, however, one should keep in mind that

Ephemeroptera are very unique and have already defied and contradicted several pillars in insect physiology (Dowse et al., 2017; Nowghani et al., 2017; Nowghani et al., 2019).

3.5.4 The effects of SCW on major haemolymph cations:

An important intracellular cation, K^+ plays an essential role in transporting other ions such a Na^+ across the basolateral membrane via NKA (Griffith, 2017). Hyperpolarizing the apical membrane, the voltage generated by VA is used to drive K^+ diffusion into cells via putative K^+ channels (Beyenbach, 2003). Additionally, at the basolateral membrane, NKA establishes an electrochemical gradient for K^+ movement into the haemolymph via basolateral K^+ channels (Jonusaite et al., 2013). Moreover, the study of K^+ flux along ionoregulatory epithelia such as the MTs and the rectum, can be indicative of the general movement of NaCl (Jonusaite et al., 2013). In the current study, we found that the entire distal apparatus of the *H. rigida* tubules reabsorb K^+, this is interesting because in other insect tubules the most distal region is a site of K^+ secretion (Berridge, 1968; Irvine, 1969; Madrell, 1969; Weng et al., 2003; Beyenbach, 2003). However, the cellular ultrastructure of the epithelial cells in the spiral region, reveal tightly packed basal folds that extend to the apical membrane, with elongated mitochondria distributed in between these folds (Nicholls, 1983). The cellular structure of this region highly resembles cells of insect rectal pads, suggesting that the cells of this region may be responsible for ion removal from the urine (Nicholls 1982). Thus, supporting this suggestion, we show that both the MTs and rectum of the FW *H. rigida* dump K^+ ions into the haemolymph. In response to the SCW, we observed a significant decline in K^+ reabsorption that was manifested along all of the distal tubule regions as well as along the rectum. This is consistent with the notion that FW insect rectum serves to conserve ions and other metabolites. If we speculate that K^+ transport is driven by NKA in the tubules, then a decrease in NKA activity may be the result of the decreased K^+ flux witnessed in the MTs, although, the same cannot be said about the rectum. However, to test this theory, future studies must be done with the inhibition of NKA to see resulting

effects in K^+ transport. Thus, for now the force behind the reduced K^+ reabsorption rates remain unknown. Furthermore, it's crucial to acknowledge the multiple factors that may be driving changes in the physiological processes of the animal such as hormones. Similar to humans, insects also produce hormones which help them acclimate to new conditions or to simply maintain their internal homeostasis. For this reason, most of the research done on tubule physiology incorporate hormonal regulation.

3.5.5 Hormone-mediated tubule secretion:

The importance of neurohormones and second messengers in short-term regulation of MT fluid secretion and ion transport has been emphasized in multiple studies (see Beyenbach, 2003 for review). However, the study of this topic in the Ephemeroptera taxa remains unexplored. To gain a better understanding on the role of MTs in achieving physiological homeostasis in the *H. rigida* nymphs, fluid secretion rates were measured. In the case where tubules do not secrete without stimulation, previous studies have demonstrated the role of 5-HT as a fluid secretion stimulator in both aquatic and terrestrial insects such as *Rhodnius prolixus* (Petzel et al., 1987; Wheelock et al.,1988) and *Aedes aegypti* (Clark and Bradleys, 1996). For example, in the aquatic larval stage of *A. aegypti*, the release of 5-HT in the haemolymph is only triggered by an incline in the haemolymph ionic load (Clark and Bradley., 1997). This is a similar situation for *H. rigida* nymphs, since exposure to SCW increases their haemolymph ionic load (Nowghani et al., 2019), therefore, leading us to speculate the release of 5-HT in mayfly nymphs. This hormone binds to its receptor, which is located on the basal membrane of principal cells that make up the tubules and increases fluid secretion in the mosquito larva through the actions of a second messenger, cAMP (Clark and Bradley, 1996). cAMP then proceeds to stimulate VA assembly on the apical membrane of principal cells, thus, initiating proton and cation secretion into the MT lumen (Rein et al., 2008). Upon observing no fluid secretion by the *H. rigida* unstimulated tubules, 5-HT was applied however, we noticed that secretion was not

stimulated. Therefore, we conclude that the likely reason for this is the absence of 5-HT receptors in *H. rigida* tubules. Besides 5-HT other diuretic hormones may be regulating fluid secretion in the mayfly nymphs (O'Donnell et al., 1996).

3.5.6 The Effects of SCW on cAMP-aided secretion:

The second messenger cAMP has been recognized as a master regulator of diuresis in a spectrum of insects (Donini et al., 2006; O'Donnell et al., 1996). This is because other peptide hormones besides 5-HT, such as CRF related or calcitonin related peptides, have the capability to activate adenylyl cyclase, causing the release of cAMP and triggering the downstream cascade that ultimately leads to diuresis (Sajadi et al., 2018). Since *H. rigida* tubules were not stimulated by 5-HT, cAMP was directly applied to the tubules and as a result *H. rigida* tubules began secreting fluid, with SCW acclimated nymph tubules secreting at a significantly higher rate than the FW nymph tubules. Furthermore, cAMP aided secretion of SCW acclimated nymph tubules produced significantly higher K^+ concentrations relative to the FW nymph tubules. A similar trend has been reported in *Ochlerotatus taeniorhynchus*, although these are seawater mosquitoes, a study was able to show rearing salinity affects the way in which cAMP behaves as a secretagogue (Donini et al., 2006). The latter study demonstrated that when the larvae were reared in 100% seawater, cAMP significantly increased K^+ concentration in the secreted fluid while decreasing Na^+ levels. Thus, we can conclude that cAMP modifies the ionic composition of the secreted fluid in *H. rigida* MTs, causing the SCW exposed nymphs to perform diuresis at a higher rate.

3.5.7 The Effects of SCW on cGMP-aided secretion:

The nitric oxide (NO) pathway has been shown to play a significant role in vertebrate and invertebrate physiology (Dow et al., 1994). The actions of nitridergic peptides in Diptera (to date only capa family known) stimulate intracellular Ca^{2+} release which then lead to cGMP production (Kean et al., 2002). Downstream targets for cGMP include protein kinase G, which, inhibits

diuresis, and protein kinase A, which, has only been shown to stimulate diuresis *in vitro* as a result of realistically unattainable high levels of cGMP (Ionescu and Donini, 2012). In insects in particular, the addition of exogenous cGMP has been shown to mediate diuresis in a stimulatory (*Drosophila*) or inhibitory (*Rhodnius, Aedes*) manner (Quinlan et al., 1997; Dow et al., 1994; Sajadi et al., 2018). Similar to what was found in *Drosophila* (Dow et al., 1994), in the current study we found that cGMP behaves like a stimulator in FW *H. rigida* tubules.

Since the stimulatory effect of cGMP had an inverse relationship with cGMP dose, we applied high doses of cAMP and cGMP together in an effort to see if cGMP could be inhibitory at high doses. Fluid secretion rates were increased in both FW and SCW tubules and this increase appeared to be greater than additive effects of cGMP and cAMP alone. This shows that cGMP, similar to *Drosophila* tubules (Dow et al., 1994), has a stimulatory effect on fluid secretion. As for the ionic composition of the secreted fluid when both secretagogues are present, in FW MTs, Na^+ concentrations go down compared to the FW tubules that were independently exposed to cAMP and cGMP, while K^+ does not alter significantly. In the SCW tubules, when competing the second messengers, the Na^+ concentration does not significantly differ from that of the messengers alone. K^+ concentrations do decline, however.

3.5.8 Ca^{2+} as an important second messenger in altering fluid secretion:

Additionally, intracellular Ca^{2+} is a key player in regulating diuresis and has been identified as a significant second messenger in adult mosquito signaling pathways (Donini et al., 2006). To confirm the involvement of Ca^{2+} as a second messenger in the mayfly nymph tubules, Ca^{2+} -ATPase inhibitor, thapsigargin was used. Thapsigargin is responsible for inhibiting Ca^{2+} ATPase, causing an intracellular accumulation of Ca^{2+}. We demonstrated that as a result of thapsigargin application, the SCW acclimated nymph tubule secretion rate was affected only at concentrations 10^{-3} M and 10^{-9} M. Furthermore, this elevation in the secretion rate is driven by the elevated Na^+ and K^+ concentrations

in the secreted fluid. Thus, it seems as though the agent responsible for releasing intracellular Ca^{2+} is specifically tailored to the high salinity environment. In both adult and larval mosquitoes, a kinin-like diuretic peptide, Leucokinin has been linked to trigger the accumulation of intracellular Ca^{2+} (Yu and Beyenbach, 2002; Donini et al., 2006). Unlike our findings, the increase in the fluid secretion rate was manifested without changes in the Na^{+} or K^{+} concentrations (Donini et al., 2006). Thus, it can be argued that in the mayfly nymph tubules, Ca^{2+} is a significant second messenger that responds to the elevated environmental salinity, however, more research must be conducted to figure out the peptide that is triggering the release of cytosolic Ca^{2+}.

3.5.9 Conclusion:

All in all, the current study has shown the profound effects of environmental salinity on the intrinsic transport mechanisms and the actions of key diuretic factors in Ephemeroptera nymphs. Contradicting many pillars in insect physiology, these nymphs fall under their own category when it comes to their possessed physiological processes. Further studies are required to better identify key transporters and cellular mechanisms that are involved in achieving intrinsic homeostasis in their natural habitat as well as in the face of contaminants such as NaCl.

3.6 References

Berridge, M. J. (1968). Urine formation by the Malpighian tubules of *Calliphora*: I. Cations. Journal of Experimental Biology, 48(1), 159-174.

Beyenbach, K. W. (2003). Transport mechanisms of diuresis in Malpighian tubules of insects. Journal of Experimental Biology, 206(21), 3845-3856.

Beyenbach, K. W. (2019). Voltages and resistances of the anterior Malpighian tubule of *Drosophila melanogaster*. Journal of Experimental Biology, 222(10), jeb201574.

Bitton, G., Jung, K., & Koopman, B. (1994). Evaluation of a microplate assay specific for heavy metal toxicity. Archives of Environmental Contamination and Toxicology, 27(1), 25-28.

Brittain, J. E. (1982). Biology of mayflies. Annual review of entomology, 27(1), 119-147.

Bradley, T. J. (1987). Physiology of osmoregulation in mosquitoes. Annual Review of Entomology, 32(1), 439-462.

Castillo, L. E., Martínez, E., Ruepert, C., Savage, C., Gilek, M., Pinnock, M., & Solis, E. (2006). Water quality and macroinvertebrate community response following pesticide applications in a banana plantation, Limon, Costa Rica. Science of the Total Environment, 367(1), 418-432.

Chasiotis, H., & Kelly, S. P. (2008). Occludin immunolocalization and protein expression in goldfish. Journal of Experimental Biology, 211(10), 1524-1534.

Clark, T. M., & Bradley, T. J. (1996). Stimulation of Malpighian tubules from larval *Aedes aegypti* by secretagogues. Journal of Insect Physiology, 42(6), 593-602.

Clark, T. M., & Bradley, T. J. (1997). Malpighian tubules of larval Aedes aegypti are hormonally stimulated by 5-hydroxytryptamine in response to increased salinity. Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America, 34(2), 123-141.

Donini, A., & O'Donnell, M. J. (2005). Analysis of Na^+, Cl^-, K^+, H^+ and NH_4^+ concentration gradients adjacent to the surface of anal papillae of the mosquito *Aedes aegypti*: application of self-referencing ion-selective microelectrodes. Journal of Experimental Biology, 208(4), 603-610.

Donini, A., Patrick, M. L., Bijelic, G., Christensen, R. J., Ianowski, J. P., Rheault, M. R., & O'Donnell, M. J. (2006). Secretion of water and ions by Malpighian tubules of larval mosquitoes: effects of diuretic factors, second messengers, and salinity. Physiological and Biochemical Zoology, 79(3), 645-655.

Dow, J. A. (1987). Insect midgut function. In Advances in insect physiology (Vol. 19, pp. 187-328). Academic Press.

Dow, J. A., Maddrell, S. H., Davies, S. A., Skaer, N. J., & Kaiser, K. (1994). A novel role for the nitric oxide-cGMP signaling pathway: the control of epithelial function in *Drosophila*. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 266(5), R1716-R1719.

Dowse, R., Palmer, C. G., Hills, K., Torpy, F., & Kefford, B. J. (2017). The mayfly nymph *Austrophlebioides pusillus* Harker defies common osmoregulatory assumptions. Royal Society Open Science, 4(1), 160520.

Del Duca, O., Nasirian, A., Galperin, V., & Donini, A. (2011). Pharmacological characterisation of apical Na^+ and Cl^- transport mechanisms of the anal papillae in the larval mosquito *Aedes aegypti*. Journal of Experimental Biology, 214(23), 3992-3999.

Griffith, M. B. (2017). Toxicological perspective on the osmoregulation and ionoregulation physiology of major ions by freshwater animals: teleost fish, Crustacea, aquatic insects, and Mollusca. Environmental toxicology and chemistry, 36(3), 576-600.

Harvey, W. R., Maddrell, S. H., Telfer, W. H., & Wieczorek, H. (1998). H^+ V-ATPases energize animal plasma membranes for secretion and absorption of ions and fluids. American Zoologist, 38(3), 426-441.

Ionescu, A., & Donini, A. (2012). AedesCAPA-PVK-1 displays diuretic and dose dependent antidiuretic potential in the larval mosquito *Aedes aegypti* (Liverpool). Journal of insect physiology, 58(10), 1299-1306.

Irvine, H. B. (1969). Sodium and potassium secretion by isolated insect Malpighian tubules. American Journal of Physiology-Legacy Content, 217(5), 1520-1527.

Jonusaite, S., Kelly, S. P., & Donini, A. (2011). The physiological response of larval *Chironomus riparius* (Meigen) to abrupt brackish water exposure. Journal of Comparative Physiology B, 181(3), 343-352.

Jonusaite, S., Kelly, S. P., & Donini, A. (2013). Tissue-specific ionomotive enzyme activity and K^+ reabsorption reveal the rectum as an important ionoregulatory organ in larval *Chironomus riparius* exposed to varying salinity. Journal of Experimental Biology, 216(19), 3637-3648.

Kaushal, S. S., Groffman, P. M., Likens, G. E., Belt, K. T., Stack, W. P., Kelly, V. R., ... & Fisher, G. T. (2005). Increased salinization of fresh water in the northeastern United States. Proceedings of the National Academy of Sciences, 102(38), 13517-13520.

Kaushal, S. S., Duan, S., Doody, T. R., Haq, S., Smith, R. M., Johnson, T. A. N., ... & Wood, K. L. (2017). Human-accelerated weathering increases salinization, major ions, and alkalinization in fresh water across land use. Applied Geochemistry, 83, 121-135.

Kean, L., Cazenave, W., Costes, L., Broderick, K. E., Graham, S., Pollock, V. P., ... & Dow, J. A. (2002). Two nitridergic peptides are encoded by the gene capability in *Drosophila melanogaster*. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 282(5), R1297-R1307.

Kefford, B. J. (2019). Why are mayflies (Ephemeroptera) lost following small increases in salinity? Three conceptual osmophysiological hypotheses. Philosophical Transactions of the Royal Society B, 374(1764), 20180021.

Kolosov, D., Piermarini, P. M., & O'Donnell, M. J. (2018). Malpighian tubules of *Trichoplusia ni*: recycling ions via gap junctions and switching between secretion and reabsorption of Na^+ and K^+ in the distal ileac plexus. Journal of Experimental Biology, 221(5), jeb172296.

Linser, P. J., Smith, K. E., Seron, T. J., & Oviedo, M. N. (2009). Carbonic anhydrases and anion transport in mosquito midgut pH regulation. Journal of Experimental Biology, 212(11), 1662-1671.

Maddrell, S. H. P. (1969). Secretion by the Malpighian tubules of *Rhodnius*. The movements of ions and water. Journal of Experimental Biology, 51(1), 71-97.

Maddrell, S. H., & O'donnell, M. J. (1992). Insect Malpighian tubules: V-ATPase action in ion and fluid transport. Journal of Experimental Biology, 172(1), 417-429.

Massaro, R. C., Lee, L. W., Patel, A. B., Wu, D. S., Yu, M. J., Scott, B. N., ... & Beyenbach, K. W. (2004). The mechanism of action of the antidiuretic peptide Tenmo ADFa in Malpighian tubules of *Aedes aegypti*. Journal of Experimental Biology, 207(16), 2877-2888.

McCormick, S. D. (1993). Methods for nonlethal gill biopsy and measurement of Na^+, K^+-ATPase activity. Canadian Journal of Fisheries and Aquatic Sciences, 50(3), 656-658.

Nicholls, S. P. (1983). Ultrastructural evidence for paracellular fluid flow in the Malpighian tubules of a larval mayfly. Tissue and Cell, 15(4), 627-637.

Nowghani, F., Chen, C. C., Jonusaite, S., Watson-Leung, T., Kelly, S. P., & Donini, A. (2019). Impact of salt-contaminated freshwater on osmoregulation and tracheal gill function in nymphs of the mayfly *Hexagenia rigida*. Aquatic Toxicology, 211, 92-104.

Nowghani, F., Jonusaite, S., Watson-Leung, T., Donini, A., & Kelly, S. P. (2017). Strategies of ionoregulation in the freshwater nymph of the mayfly *Hexagenia rigida*. Journal of Experimental Biology, 220(21), 3997-4006.

O'Donnell, M. (2008). Insect excretory mechanisms. Advances in Insect Physiology, 35, 1-122.

O'Donnell, M. J., Dow, J. A., Huesmann, G. R., Tublitz, N. J., & Maddrell, S. H. (1996). Separate control of anion and cation transport in malpighian tubules of *Drosophila Melanogaster*. Journal of Experimental Biology, 199(5), 1163-1175.

Ontario's Great Lakes Strategy 2016 Progress Report:
https://www.ontario.ca/page/ontarios-great-lakes-strategy-2016-progress-report

Petzel, D. H., Berg, M. M., & Beyenbach, K. W. (1987). Hormone-controlled cAMP-mediated fluid secretion in yellow-fever mosquito. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 253(5), R701-R711.

Phillips, J. (1981). Comparative physiology of insect renal function. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 241(5), R241-R257.

Pollock, V. P., McGettigan, J., Cabrero, P., Maudlin, I. M., Dow, J. A., & Davies, S. A. (2004). Conservation of capa peptide-induced nitric oxide signaling in Diptera. Journal of Experimental Biology, 207(23), 4135-4145.

Quinlan, M. C., Tublitz, N. J., & O'Donnell, M. J. (1997). Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stål: the peptide CAP2b and cyclic GMP inhibit Malpighian tubule fluid secretion. Journal of Experimental Biology, 200(17), 2363-2367.

Ramsay, J. A. (1954). Active transport of water by the Malpighian tubules of the stick insect, *Dixippus Morosus* (Orthoptera, Phasmidae). Journal of Experimental Biology, 31, 104-113.

Rein, J., Voss, M., Blenau, W., Walz, B., & Baumann, O. (2008). Hormone-induced assembly and activation of V-ATPase in blowfly salivary glands is mediated by protein kinase A. American Journal of Physiology-Cell Physiology, 294(1), C56-C65.

Sajadi, F., Curcuruto, C., Al Dhaheri, A., & Paluzzi, J. P. V. (2018). Anti-diuretic action of a CAPA neuropeptide against a subset of diuretic hormones in the disease vector *Aedes aegypti*. Journal of Experimental Biology, 221(7), jeb177089.

Water Quality in Ontario 2014 Report:
https://www.ontario.ca/page/water-quality-ontario-2014-report#foot-xxxiii

Weng, X. H., Huss, M., Wieczorek, H., & Beyenbach, K. W. (2003). The V-type H^+-ATPase in Malpighian tubules of *Aedes aegypti*: localization and activity. Journal of Experimental Biology, 206(13), 2211-2219.

Willmer, P. (2006). Osmoregulation in Invertebrates. Encyclopedia of Life Sciences.

Yu, M. J., & Beyenbach, K. W. (2002). Leucokinin activates Ca^{2+}-dependent signal pathway in principal cells of *Aedes aegypti* Malpighian tubules. American Journal of Physiology-Renal Physiology, 283(3), F499-F508.

Zadeh-Tahmasebi, M., Bui, P., & Donini, A. (2016). Fluid and ion secretion by Malpighian tubules of larval chironomids, *Chironomus riparius*: effects of rearing salinity, transport inhibitors, and serotonin. Archives of Insect Biochemistry and Physiology, 93(2), 67-85.

Zall, D. M., Fisher, D., & Garner, M. Q. (1956). Photometric determination of chlorides in water. Analytical Chemistry, 28(11), 1665-1668.

CHAPTER 4:

SUMMARY AND FUTURE DIRECTION

4.1 Perspectives and future directions:

Around the 1930s and 1940s, the study of aquatic insects stemmed from an interest in fishing (Merrit et al., 2008); however, as time has passed aquatic insects have been heavily utilized in ecosystem and ecology research, mostly as water quality bio-indicators (Cummins, 1973; Melo, 2009; Monteiro-Júnior et al., 2013). One of the most important insect orders in aquatic ecosystems is the Ephemeroptera (the mayfly) nymphs, mostly due to their abundance in most aquatic freshwater regions (Barber-James et al., 2008). Found in fossils dating back to the Lower Permian Period, mayflies have been recognized as the oldest extant winged insects (Hubbard and Kukalová-Peck, 1980). Therefore, it has been alarming to witness local extinctions of certain mayfly species within the past few decades (Da-Silva and Salles 2012). It is no coincidence that changes in mayfly abundance as well as extirpation events are concurrent with reported accumulation of pollutants in FW bodies (Da-Silva and Salles 2012). In fact, there has been a growing interest in the study of mayflies as biologic indicators of water quality due to this reason (Howland et al., 2019; Yanygina and Yevseyeva, 2018; Paparisto et al., 2009; Kovats and Ciborowski, 1989). Therefore, the significant role of mayfly nymphs in reflecting and informing us on water and sediment toxicity is widely acknowledged in the fields of ecology and toxicology. Studies have focused on measuring the survivability of mayflies when exposed to pollutants and determining lethality thresholds. However, there remains a gap in the field of physiology, since very little research has been done on mayflies as model organisms in this field. In hopes of filling in this gap, as a master's student I continued to explore the physiology of specific mayfly nymph osmoregulatory organs in response to environmental contaminants such as NaCl. The major findings of the specific objectives outlined in chapter 1 are summarized below.

4.2 Impact of NaCl on physiological endpoints of ion and water balance:

In chapter 2, it was shown that the major haemolymph ions levels were significantly increased in *H. rigida* nymphs exposed to salt-contaminated water (SCW) for a period of 7 days as well as an elevation in body water content. Further studies conducted in chapter 3 revealed that in addition to the elevated haemolymph ion concentrations, the haemolymph osmolality also increased significantly in response to SCW. In both FW and SCW environments, the nymphs maintained their haemolymph osmolality slightly higher than the external environment. Put together with other data collected, I have shown that high salinity conditions impose a serious risk on haemolymph homeostasis, resulting to changes in ionoregulatory epithelial function to compensate for the excess ions.

4.3 Impact of NaCl on ionocyte ultrastructure and function:

The tracheal gills of mayfly nymphs were first recognized as respiratory organs, which came as no surprise since the gills are in fact external extensions of the tracheal system (Dodds and Hisaw, 1924; Wingfield, 1939). Later on, Wichard and Komnick (1971), reported the presence of specialized salt transporting cells called "Ephemerid chloride cells" (referred to as its modern name 'ionocytes' in this thesis), similar to cells discovered in teleost gill epithelia (Keys and Willmer, 1932). With the limitation of technology at the time, a few studies were published, speculating about the ionoregulatory role of the tracheal gills (Saouter et al., 1991; Filshie and Campbell, 1984; Wichard et al., 1973; Komnick and Stockem, 1973). Only recently direct functional evidence of the ionoregulatory role of the ionocytes present on the tracheal gills has been reported (Nowghani et al., 2017). The results in chapter 2 showed that in FW conditions the tracheal gills absorbed Na^+ from the surrounding water, whereas in the SCW, the gills secreted Na^+. Interestingly, the observed change in ion transport was not driven by the active transport pathway or ionocyte remodeling, but concurrent with structural changes in the paracellular pathway. Overall, this chapter provides a novel look into the osmoregulatory mechanisms used by *H. rigida* nymphs to tolerate FW salinization.

4.4 Impact of SCW on the ionoregulatory function of the MTs and rectum:

Besides the tracheal gills, the excretory system in insects, comprised of the MTs and the hindgut, play an essential role in achieving homeostasis. Chapter 3 provides the first look into the ionoregulatory function of *H. rigida* MTs and rectum, which can be used to better understand the overall physiological response of the nymphs to SCW. Using SIET, K^+ (an important ionoregulatory ion) fluxes were measured along the length of the MTs and the rectal portion of the hindgut, revealing K^+ reabsorption along all measured regions. In response to SCW, the measured regions continued to reabsorb K^+, however at a lower rate. To isolate the role of tubules in achieving haemolymph homeostasis, fluid secretion was measured in the presence of diuretic factors. The results revealed that the tubules do not secrete *in vitro* unless stimulated by diuretic factors and highlighted the momentous impact of environmental salinity on the effects of these factors in the process of urine formation.

4.5 Future directions:

The study of FW contamination with NaCl has become an important area of research as water salinity in certain regions are increasing annually at alarming rates due to anthropogenic activities. Additionally, mayfly nymphs have been used in ecological and toxicological testing many years with minimal data available on their physiological mechanisms. The present studies have provided insight into salt and water balance of FW mayfly nymphs, acting as a link between the field of physiology and ecology. This has been achieved by demonstrating that important ionoregulatory organs such as the MTs, hindgut and tracheal gills will significantly alter their function to acclimate to changes in environmental salinity.

Future studies into the paracellular pathway via septate junction complexes that are altered in tracheal gills of SCW-exposed nymphs, can provide better insight into the coping mechanisms practiced by *H. rigida* nymphs. For example, in *Aedes aegypti*, specific claudin-like septate junction

proteins, Megatrachea (Mega) and Kune-kune (Kune) have been reported in ectoderm derived epithelium such as the hindgut and anal papillae (Jonusaite et al., 2016). After exposure to 30% seawater, changes in changes in Mega transcript and Kune protein abundance in the anal papillae highlights the role of this organ in coping with fluctuating environmental salinity. Two other septate junction proteins, snakeskin (Ssk) and mesh have also been reported in *A. aegypti* endoderm derived organs, which include the gut and the MTs. With alterations in both Ssk and mesh abundance in response to 30% seawater, the function of these proteins was suggested to be linked with Cl^- permeability in the threat of increased ambient salinity, which also imposes a leakier epithelium (Jonusaite et al., 2017). In the same light, it would be worth looking at specific junction proteins which may be driving the changes in mayfly gill epithelium and ion transport.

Additionally, further investigation into the mechanisms behind other cAMP triggering peptide hormones such as corticotropin-related factor (CRF) diuretic peptide and a calcitonin-related diuretic hormone, which have been discovered in other insects like *A. aegypti*, can provide more insight into ion regulation in *H. rigida* nymphs (Sajadi et al., 2018). Moreover, the use of inhibitors such as bafilomycin and Ouabain to limit ATPase activity in the MTs or the rectum concurrent with the flux measurement of major ionoregulatory ions such as Na^+ and Cl^- can provide a more comprehensive look into the coping mechanisms of *H. rigida* when faced with salt contamination.

4.6 References

Barber-James, H. M., Gattolliat, J. L., Sartori, M., & Hubbard, M. D. (2007). Global diversity of mayflies (Ephemeroptera, Insecta) in freshwater. In Freshwater Animal Diversity Assessment (pp. 339-350). Springer, Dordrecht.

Cummins, K. W. (1973). Trophic relations of aquatic insects. Annual review of entomology, 18(1), 183-206.

Da-Silva, E. R., & Salles, F. F. (2012). Ephemeroptera Hyatt & Arms, (1891). Insetos do Brasil: Diversidade e taxonomia. Ribeirão Preto, Holos, 231-244.

Dodds, G. S., & Hisaw, F. L. (1924). Ecological studies of aquatic insects: size of respiratory organs in relation to environmental conditions. Ecology, 5(3), 262-271.

Filshie, B. K., & Campbell, I. C. (1984). Design of an insect cuticle associated with osmoregulation: the porous plates of chloride cells in a mayfly nymph. Tissue and Cell, 16(5), 789-803.

Howland, J. R., Alexander, A. C., Milani, D., Culp, J. M., & Peru, K. M. (2019). Effects of oil sands process water mixtures on the mayfly *Hexagenia* and field-collected aquatic macroinvertebrate communities. Ecotoxicology, 1-11.

Hubbard, M. D., & Kukalová-Peck, J. (1980). Permian mayfly nymphs: new taxa and systematic characters. In Advances in ephemeroptera biology (pp. 19-31). Springer, Boston, MA.

Jonusaite, S., Kelly, S. P., & Donini, A. (2016). The response of claudin-like transmembrane septate junction proteins to altered environmental ion levels in the larval mosquito *Aedes aegypti*. Journal of Comparative Physiology B, 186(5), 589-602.

Jonusaite, S., Donini, A., & Kelly, S. P. (2017). Salinity alters snakeskin and mesh transcript abundance and permeability in midgut and Malpighian tubules of larval mosquito, Aedes aegypti. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 205, 58-67.

Keys, A., & Willmer, E. N. (1932). 'Chloride secreting cells' in the gills of fishes, with special reference to the common eel. The Journal of physiology, 76(3), 368-378.

Komnick, H., & Stockem, W. (1973). The porous plates of coniform chloride cells in mayfly larvae: High-resolution analysis and demonstration of solute pathways. Journal of cell science, 12(3), 665-681.

Kovats, Z. E., & Ciborowski, J. J. (1989). Aquatic insect adults as indicators of organochlorine contamination. Journal of Great Lakes Research, 15(4), 623-634.

Melo, A. S. (2009). Explaining dissimilarities in macroinvertebrate assemblages among stream sites using environmental variables. Zoologia, 26(1).

Merritt, R.W., Cummins, K.W. & Berg, M.B. (2008). An introduction to the aquatic insects of North America. Kendall Hunt.

Monteiro Júnior, C., Couceiro, S. R. M., Hamada, N., & Juen, L. (2013). Effect of vegetation removal for road building on richness and composition of Odonata communities in Amazonia, Brazil. International Journal of Odonatology, 16(2), 135-144.

Nowghani, F., Jonusaite, S., Watson-Leung, T., Donini, A., & Kelly, S. P. (2017). Strategies of ionoregulation in the freshwater nymph of the mayfly *Hexagenia rigida*. Journal of Experimental Biology, 220(21), 3997-4006.

Paparisto, A., Halimi, E., Keçi, E., Hamzaraj, E., & Laknori, O. (2009). using insects as bio-indicators to assess water quality of Albanian rivers. Journal of International Environmental Application & Science, 4(3), 246-252.

Sajadi, F., Curcuruto, C., Al Dhaheri, A., & Paluzzi, J. P. V. (2018). Anti-diuretic action of a CAPA neuropeptide against a subset of diuretic hormones in the disease vector *Aedes aegypti*. Journal of Experimental Biology, 221(7), jeb177089.

Saouter, E., Le Menn, R., Boudou, A., & Ribeyre, F. (1991). Structural and ultrastructural analysis of gills and gut of *Hexagenia rigida* nymphs (Ephemeroptera) in relation to contamination mechanisms. Tissue and Cell, 23(6), 929-938.

Wichard, W., & Komnick, H. (1971). Electron microscopial and histochemical evidence of chloride cells in tracheal gills of mayfly larvae. Cytobiologie, 3(2), 215-228.

Wichard, W., Tsui, P. T. P., & Komnick, H. (1973). Effect of different salinities on the coniform chloride cells of mayfly larvae. Journal of Insect Physiology, 19(9), 1825-1835.

Wingfield, C. A. (1939). The function of the gills of mayfly nymphs from different habitats. Journal of Experimental Biology, 16(3), 363-373.

Yanygina, L. V., & Yevseyeva, A. A. (2018). Impact of mining on macroinvertebrate species richness in mountain streams. Acta Biologica Sibirica, 4(3), 69-74.